ApoE2-Mediated Neuroprotective Mechanism Through Up-regulation of Glycolysis

By

Xin Zhang

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Chair: Liqin Zhao, Ph.D.

Rick Dobrowsky, Ph.D.

Eduardo Rosa-Molinar, Ph.D.

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The Thesis Committee for Xin Zhang certifies that this is the approved version of the following thesis:

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Chair, Liqin Zhao, Ph.D.

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Abstract

Studies presented here aim at gaining an insight into mechanisms of how human apolipoprotein E (ApoE) isoforms impact glucose metabolism, particularly through regulation of glycolysis, which may ultimately result in pathophysiological alterations in the brain. Consistent with our previous findings, hexokinase, the enzyme that catalyzes the initial and irreversible conversion of glucose to 6-phophoglucose in the first step of glycolysis, is significantly affected by ApoE isoforms in stably human Apo E2, 3, or 4 expressing Neuro-2a cell lines. Results from a time course study indicate that the regulation of hexokinase by ApoE is typically through a chronic pattern. Additionally, glycolytic function was also differentially regulated by three ApoE isoforms with ApoE2 group shows the most robust profile. The data indicate that hApoE2-expressing cells exhibited significantly enhanced glycolytic activity compared to ApoE4-expressing cells, possibly through the upregulation of hexokinase.

With the evidence that ApoE isoforms differentially regulate glycolytic function via hexokinase, cell health status was further assessed by both metabolic activity and morphological phenotype. In line with our prediction, the regulation of hexokinase and the differential glycolytic profiles directly correlated to the overall health status of the three ApoE isoforms-expressing cells. Meanwhile, we observed no significant alteration in apoptotic markers and the insulin-regulated glucose transporter, which further supports a neuroprotective role of ApoE2 through up-regulation of glucose metabolism. Furthermore, this thesis employed a differentiated neuronal model to determine the influence of ApoE on the regulation of neuronal glycolysis. In transfected neurons, differential regulation of hexokinase and glycolytic function by hApoE2/3/4 was also observed. hApoE2-transfected

neurons exhibited a significantly higher expression and activity of hexokinase as well as lactate production than cells transfected with ApoE3 or ApoE4. Taken together, results from these studies indicate that human ApoE isoforms differentially modulate neuronal glycolysis via regulation of hexokinase, which directly correlates to neuronal metabolic activity and health status. The ApoE2-mediated glycolytic robustness may suggest a mechanistic rationale for its neuroprotective role and consequently provides a novel therapeutic approach against the onset of AD.

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Thank you

Xin Zhang

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List of Abbreviations

2-DG	2-Deoxy-D-glucose
ABCG1	ATP-binding cassette sub-family G member 1
ABCG4	ATP-binding cassette sub-family G member 4
AD	Alzheimer's disease
AG	aerobic glycolysis
ApoE	apolipoprotein E
APP	Amyloid precursor protein
Aβ	amyloid beta
BAX	Bcl-2-associated X protein
BBB	blood brain barrier
CMRglc	cerebral glucose metabolic rate
CNS	central nervous system
CSF	cerebrospinal fluid
ECAR	extracellular acidification rate
EPR	electron paramagnetic resonance
EPSC	excitatory postsynaptic current
FC	fold change
FDG-PET	[^{18}F] fluorodeoxyglucose-positron emission tomography
FRET	fluorescence resonance energy transfer
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
GLUTs	facilitated glucose transporters

GSK-3	Glycogen synthase kinase-3
hApoE-TR	human ApoE gene-targeted replacement mice
HDL	high-density lipoproteins
HK1	hexokinase I
HK2	hexokinase II
Igf1	insulin-like growth factor 1
IRAP	insulin-regulated aminopeptidase
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LDLR	Low-Density Lipoprotein (LDL) Receptor
LOAD	late-onset AD
LRP	LDL-receptor-related protein
MCI	mild cognitive impairment
O-GlcNAc	O-linked β-N-acetylglucosamine
Pfk1p	yeast phosphofructokinase-1 subunits
RA	retinoic acid
v-ATPase	Vacuolar-type H + -ATPase
VLDL	very low-density lipoprotein

Purpose of this Thesis

Alzheimer's disease (AD) composes a major part of dementia and imposes a great economic burden globally. To date, AD, the dominant dementia, is the only top 10 cause of death in the United States that cannot be prevented, cured, or even slowed (Association, 2018). By 2040, the number of individuals having dementia is expected to increase by 190% in Europe, North America, and the developed Western Pacific regions (Hampel et al., 2011). Some speculate that if we could manage to delay the onset of AD by 50%, the number of AD dementia diagnosis would be reduced by nearly half (R. A. Sperling et al., 2011). For the past two decades, almost all the clinical trials have failed in finding a treatment that could slow or halt the progression of AD (Khan, 2018), underscoring the need of a novel approach that focuses less on the pathological mechanism of the disease but more on neuroprotective mechanism that would promote brain resilience against the onset of AD. Converging evidence indicates that pathophysiological changes in the brain occur decades before the onset of Alzheimer's clinical symptoms. This preclinical and chronic progression of AD, also known as the "prodromal" stage, would provide us a potential opportunity for therapeutic intervention by targeting early changes in the brain that help slow down synaptic loss and neuronal death. Recent studies suggest that bioenergetic dyshomeostasis plays an important role in the earliest stage of AD (preclinical), especially through dysfunction of glycolysis that possibly leads to ultimate cognitive decline and memory loss (An et al., 2018; Cunnane et al., 2011; Hoyer, Oesterreich, & Wagner, 1988; Lying-Tunell, Lindblad, Malmlund, & Persson, 1981; Sims et al., 1980; Vlassenko et al., 2018; Vlassenko & Raichle, 2015). Higher brain glucose and reduced glycolytic flux are also related to the severity of AD, with more plaques and

tangles found in the brains of Alzheimer's patients (An et al., 2018). Therefore, it is imperative to better understand whether and how this abnormal energy metabolism, specifically glycolysis, would potentially impact the development of AD. This understanding may, provide a novel therapeutic intervention to prevent the onset and/or the progression of the disease from its preclinical stage to the clinical stage.

Carrying apolipoprotein gene E4 (ApoE4) alleles is one of the greatest risk factors for the development of late onset AD. Studies indicate a reduced cerebral metabolism of glucose in individuals carrying ApoE4 (Reiman et al., 2004, 2005). Previously, our lab reported that human ApoE2 gene-targeted (hApoE2-TR) brain exhibited the most robust gene expression whereas human ApoE4 gene-targeted (hApoE4-TR) brain displayed the most deficient gene expression profile in glucose uptake and glycolytic pathway. Whether and how this regulation would impact the overall metabolic activity and neuronal health status, however, remained unclear. In the present study, we aimed at confirming the role of human ApoE in regulating glycolytic function via hexokinase in both human ApoE stable cell lines and neuronal model as well as whether this regulation would further impact overall cell health status and metabolic activity. We hypothesized that ApoE2-mediated neuroprotective mechanism functions through the up-regulation of glycolysis via hexokinase.

Introduction

Current status and challenges of Alzheimer's disease

Alzheimer's disease (AD), a neurodegenerative disease characterized by gradual loss of memory and cognitive activities, is the most common cause of dementia affecting more than 5 million Americans; by 2050, this number could rise up to 14 million. AD ranks as the sixth-leading cause of death in the United States and the fifth-leading cause of death among those aged 65 and older. AD and other dementias cause a great economic burden, which will cost the nation $277 billion in 2018. An overall estimated medical and caregiving cost for AD might be $1.1 trillion by 2050 (Association, 2018).

For the past thirty years, the accumulation of toxic amyloid beta (Aβ) has been considered the central player in the development of AD, since it correlated with gene (APP, PSEN1 and PSEN2) mutations that lead to abnormal amyloid precursor protein (APP) breakdown and generation of Aβ (Reitz & Mayeux, 2014) . It is evidenced by a great amount of studying on early onset AD (EOAD, onset < 65 years). However, this Aβ hypothesis, is not sufficient to explain the pathological changes in late-onset AD (LOAD, onset ≥ 65 years), since there are no specific mutations that are associated with the inheritance of LOAD (Awada, 2015), and accounts for about 95% of the cases of AD. Several risk factors for the development of LOAD have been identified during past decades, of which aging is thought to be the greatest. Others include the presence of one or more ApoE4 allele s, low educational and occupational attainment, family history of AD, moderate or severe traumatic brain injuries, and cardiovascular risk factors (Apostolova, 2016). For the past 100 years, the exact pathogenesis of AD has remained unclear and no

cure has yet been found. Some medication exists, but the benefits are small. Further, none of the medications can clearly and definitively halt the progression of AD (J. Birks & Harvey, 2006; J. S. Birks & Grimley Evans, 2015; "Drugs for Alzheimer's disease: best avoided. No therapeutic advantage," 2012). Studies have revealed that pathophysiological changes in the brain occur decades before the onset of Alzheimer's clinical symptoms. If there could be an effective therapeutic intervention during this phase that delays the onset of AD, the overall projected number of individuals having AD would likely decrease by 38% in 2050 (Sloane et al., 2002). Therefore, it is imperative that we gain a better understanding of the risk mechanism of AD and develop a novel approach that leverages the brain's self-defense mechanism to increase its resilience against the development of AD.

Biochemical and structure properties of apolipoprotein E

Apolipoproteins are a group of proteins that bind lipid or cholesterol to form lipoproteins. Lipoproteins then enable water-insoluble substances to transfer through the lymphatic and circulatory systems. The three-major human ApoE alleles (ε2, ε3 and ε4) arose after the primate-human split around 7.5 million years ago. A comprehensive analysis of haplotype diversity showed that ε4 is the ancestral ApoE allele in human (Fullerton et al., 2000). About 220,000 years ago, the ε3 allele emerged after an arginine was substituted with a cysteine in the ApoE4 gene at residue 112 (Arg112Cys). ApoE2 is the youngest family member and was created after another substitution of an arginine to cysteine took place at residue 158 (Arg158Cys) 80,000 years ago (Huebbe & Rimbach, 2017; Weisgraber, Rall, & Mahley, 1981). Liver and brain are the two major organs that synthesize ApoE, with the liver production accounting for more than 75% of total ApoE

(Elshourbagy, Liao, Mahley, & Taylor, 1985). In the peripheral system, ApoE is highly expressed in hepatocytes and peritoneal macrophages. It is responsible for transporting liver-synthesized very low-density lipoproteins (VLDL) and intestine-synthesized chylomicrons. Despite some of the smaller circulating HDL lipoproteins, plasma ApoE cannot cross the BBB, thus ApoE in the CNS must be produced locally.

In the brain, ApoE is predominantly synthesized by astrocytes, and to some extent by microglia, vascular smooth muscle cells and the choroid plexus (Pitas, Boyles, Lee, Foss, & Mahley, 1987; Uchihara et al., 1995; Q. Xu et al., 2006). The synthesis and secretion of ApoE were also observed in some neurons, especially under stress conditions like traumatic brain injury, although at a lower level than in astrocytes (Lahiri & Maloney, 2009; Petegnief, Saura, de Gregorio-Rocasolano, & Paul, 2001). ApoE also promotes neurite outgrowth, neuronal sprouting and synapse formation via LDL receptors (Giau, Bagyinszky, An, & Kim, 2015). Interestingly, nascent astrocyte-produced particles contain little core lipid, while CSF lipoproteins are the size and density of plasma HDL, contain the core lipid, esterified cholesterol, and are spherical indicating a possible post-secreted modification before ApoE reaches the CSF (LaDu et al., 1998). In order to be stable in the CNS, ApoE requires proper lipidation, as evidenced by a significant reduction of ApoE level in the brain when the murine Abca1 gene was deleted (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004) ; the Abca1 gene codes for a cholesterol transported.

Human ApoE is a polymorphic protein whose gene locates to chromosome 19q13.2 and contains 4 exons (Mahley, 1988). Complete amino acid sequencing revealed that two residues distinguishing the three ApoE isoforms are located at residues 112 and 158. ApoE2 has Cys residue at both sites. ApoE3 has a Cys residue at 112 and Arg residue at

158. ApoE4 has Arg residues at both sites (Rall, Weisgraber, & Mahley, 1982; Weisgraber et al., 1981) (Fig. 1).

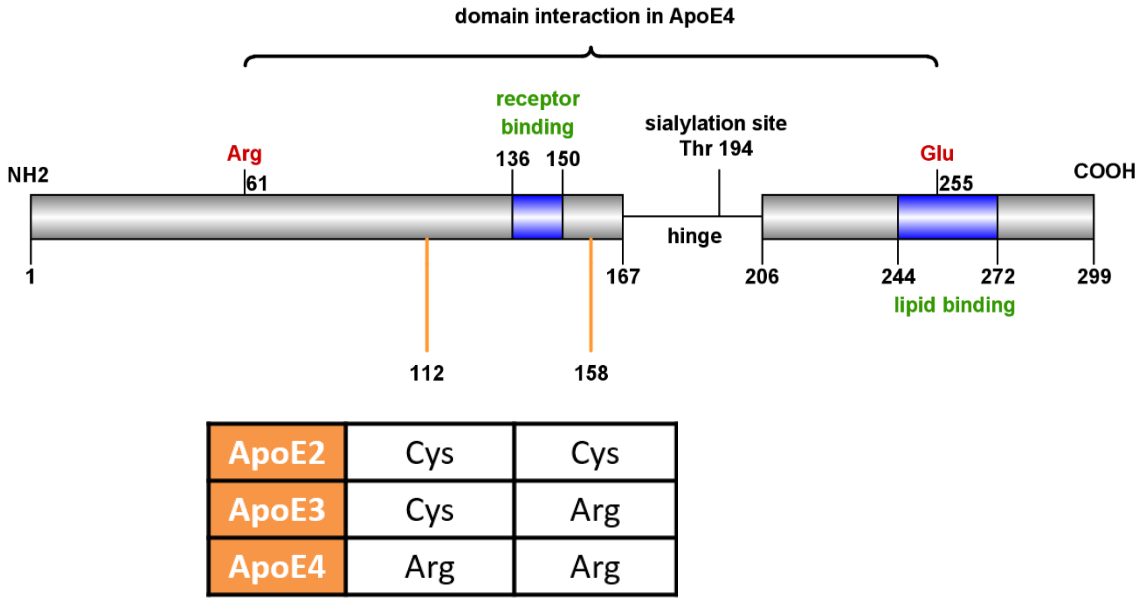


Figure 1. Schematic illustration of human ApoE. Human apolipoprotein E (ApoE) is a glycosylated protein containing 299 amino acids. Two main functional domains separated by a protease-sensitive hinge region are shown above: N-terminal domain (residue 1-167) contains a receptor binding region (residue 136-150). C-terminal domain (residue 206-299) contains a lipid binding region (residue 244-272). Residues distinguish three ApoE isoforms locate at 112 and 158. ApoE2 has Cys residue at both sites. ApoE3 has a Cys residue at 112 and Arg residue at 158. ApoE4 has Arg residues at both sites (Rall, Weisgraber, & Mahley, 1982; Weisgraber et al., 1981). Predicted interaction of Arg 61 and Glu 255 in ApoE4 is indicated. A sialylation position in ApoE, Thr 194, is also presented, yet how glycosylation/sialylation of ApoE affects its metabolic function remained unclear.

The minor difference at 112 and 158 among three ApoE isoforms might result in significant, perhaps profound changes in structures and biological properties. ApoE has two functional domains linked by a protease-sensitive hinge region. Different interaction of these two functional domains differentiates ApoE4 from ApoE2/3 (Dong & Weisgraber, 1996; Dong et al., 1994). Arg-112 reduces protein stability or molten globule formation mediated by

ApoE4 and is considered to contribute to ApoE4-associated neuropathology (Morrow et al., 2002; Weisgraber, 1990). X-ray crystallographic studies indicate that due to the salt bridge formed by Arg-112 and Glu-109, Arg-61 was extended away from the four-helix bundle in ApoE4, whereas the side chain of Arg-61 in ApoE3 and ApoE2 has a different orientation (tucked between helices 2 and 3) (Dong et al., 1994; Wilson, Wardell, Weisgraber, Mahley, & Agard, 1991). Weisgraber et al. suggested that Arg-61 in ApoE4 interacts with Glu-255 in the C-terminal region (Weisgraber, 1990) (Fig. 2). There is also evidenced by fluorescence resonance energy transfer (FRET) and electro-paramagnetic resonance (EPR) which indicate a closer distance between Arg-61 and Glu255 (Hatters, Budamagunta, Voss, & Weisgraber, 2005).

The unique structure of ApoE4 indicates its key role in stimulating accumulation and clearance of amyloid β. Fragments of ApoE is suggested to be detrimental within the cell (Y. Huang et al., 2001). Studies indicate that ApoE4 is highly susceptible to proteolysis when compared to ApoE3 (Brecht et al., 2004; F. M. Harris et al., 2003). Intriguingly, by mutating Arg-61 to threonine or Glu-255 to alanine, the susceptibility of ApoE4 to proteolysis is significantly reduced (Mahley, Weisgraber, & Huang, 2006). Disrupting domain interaction near Arg-61 and Arg-112 has also been reported to diminish the effect of ApoE4, but not ApoE3 (Bales et al., 1999; Holtzman et al., 2000; LaDu et al., 1994; Ye et al., 2005). Based on these findings, a novel intervention was proposed in which a small molecule that was predicted to interfere with the domain interaction mediated at Arg-61 in ApoE4 would convert ApoE4 to “ApoE3-like” molecule, thus potentially abolishing the negative effect by ApoE4 in AD (Mahley et al., 2006) (Fig. 2).

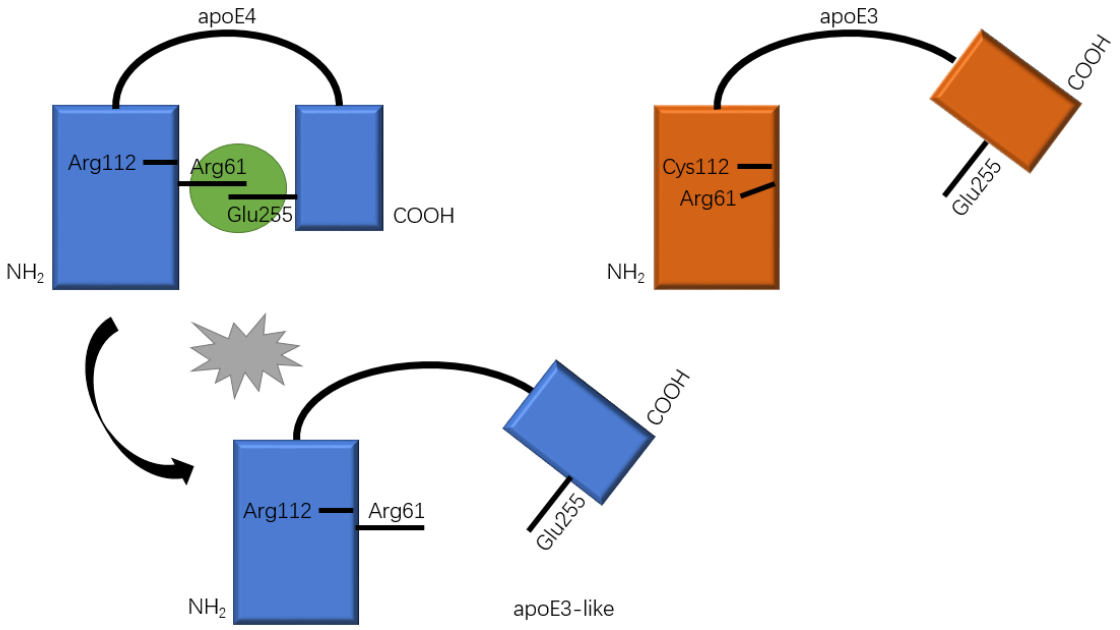


Figure 2. A small molecule (indicated by grey symbol) predicted to interfere the domain interaction mediated at Arg-61 in ApoE4 would convert ApoE4 to "ApoE3-like" molecule. In ApoE4, Arg-61 interacts with Glu-255, but in ApoE3, it does not so. By interrupting the domain interaction using a small molecule would convert ApoE4 to "ApoE3-like" molecule (Mahley et al., 2006).

Receptor- and lipid-binding affinities are also dramatically different among the three ApoE isoforms. ApoE2 has a significantly lower affinity for LDL receptors compared to ApoE3/4 (Schneider et al., 1981). A mechanism underlying the lower affinity of ApoE2 for the LDLR in is reported to correlate to the Cys 158 forming a new salt bridge between Arg 150 and Asp 154, which is probably also the cause of a genetic disorder termed type III hyperlipoproteinemia (Dong et al., 1994; Hatters, Peters-Libeu, & Weisgraber, 2006).

Glycosylation/sialylation is a particularly common, yet complex and important post-translational modification of secreted and cell surface glycoprotein. Human ApoE exists as multiple glycosyllated and sialylated glycoforms with 80%-85% of plasma ApoE

being in an asialo form. Interestingly, in the CNS, ApoE is more highly glycosylated/sialylated than in plasma. Multiple sialylations can be caused at multiple sites containing one ApoE sugar linkage or one site with several sialic acid residues (Zannis & Breslow, 1981). Carbohydrate residues are attached to ApoE via an O-linkage to Thr194.Variable post-translational O-linked glycosylation/sialylation at Thr194 was found to contribute to the charge difference among three ApoE isoforms (Wernette-Hammond et al., 1989; Zannis & Breslow, 1981). It is indicated that newly-synthesized ApoE is highly sialylated (Wernette-Hammond et al., 1989). In the CNS, ApoE is reported to play an important role in the mobilization and reutilization of lipid in the repair, growth, and maintenance of myelin and axonal membranes (Ignatius et al., 1986; Skene & Shooter, 1983; Snipes, McGuire, Norden, & Freeman, 1986). In mice transgenic for human ApoE2/3/4 alleles, sialylated ApoE isoproteins are preferentially associated with neurons and contribute significantly (50%-60%) to the total neuronal ApoE in neuronal cultures, indicating it may be a major resource for ApoE during development or after nerve injury (Xu et al., 1999). Glycosylation defects in APP, tau and other proteins also have been reported in AD. Multiple dysfunctional proteins related to AD due to defective glycosylation were suggested as well, including butyrylcholinesterase, cholinesterase, neural cell adhesion molecule, v-ATPase, and tyrosine-related kinase B (Schedin-Weiss, Winblad, & Tjernberg, 2014). The role that sialylated ApoE plays in neuronal metabolism and AD remains to be determined.

Apolipoprotein E and AD

In the central nervous system, astrocytes are responsible for the synthesis and assembly of lipoproteins. However, emerging evidence showed that neurons are also

involved in synthesis and regulation of lipid metabolism in the brain, at least for cholesterol (Pfrieger & Ungerer, 2011). Cholesterol, which is essential in the membrane as well as myelin sheaths, is transferred under the facilitation of ApoE from the astrocytes to neuron. It also plays an important role in synaptogenesis and maintaining proper synaptic function (Pfrieger, 2003). ApoE is transported by ATP-binding cassette (ABC) transporters, such as ABCA1 and ABCG1 in astrocytes, while ABCG4 is only in neurons (Vance & Hayashi, 2010). ApoE can also promote axonal growth and neuronal survival through interactions with the LDLR superfamily on neurons (Hayashi, Campenot, Vance, & Vance, 2007). Most recently, much evidence has demonstrated the interaction between ApoE and Aβ in ε4 carriers accelerates the deposition and production of Aβ compared to noncarriers (Kok et al., 2009; Polvikoski et al., 1995; Reiman et al., 2009). ApoE4 is identified as one of the greatest risk factors for developing LOAD as evidenced by not only a great prevalence of AD, but also an early onset and altered response to AD treatment (Corder et al., 1993; C. C. Liu, Liu, Kanekiyo, Xu, & Bu, 2013). ApoE2, on the other hand, has been generally reported to be neuroprotective and slow down the progression of AD. ApoE2 carriers without dementia tend to have less amyloid pathology and do not display typical increases in age-related Aβ burden (Grothe, Villeneuve, Dyrba, Bartres-Faz, & Wirth, 2017). Amygdala functional connectivity is also more likely to remain stable in ApoE2 carries (Gong et al., 2017) . Several studies have suggested that increased functional connectivity in the entorhinal cortex in individuals having at least one ApoE2 allele (ε2/ε2 or ε2/ε3), potentially underlies the mechanism of improved episodic memory performance (Craft et al., 1998; Martins, Oulhaj, de Jager, & Williams, 2005; Shinohara et al., 2016). Moreover, structural integrity is also positively impacted by ApoE2 since a lower degree of age-

related myelin breakdown (Bartzokis et al., 2006) and attenuated hippocampal atrophy was observed in individuals having ApoE2 compared to noncarriers (Chiang et al., 2010).

Perturbed energy metabolism, hexokinase and AD

Much evidence supports that pathophysiological alterations occurs in a preclinical stage of AD long before the clinical symptoms of Alzheimer's disease are manifest. During this stage, one of the striking pathological features is the severe reduction of glucose utilization in the brain (de Leon et al., 2001; Langbaum et al., 2009; L. Mosconi, S. De Santi, J. Li, et al., 2008; Mosconi et al., 2006; Small et al., 1995). Positron emission tomography (PET) imaging with 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG) as the tracer has been used to measure the qualitative and quantitative change of cerebral metabolic rate of glucose (CMRglc) in preclinical stage of AD. CMRglc is also considered to be a critical indicator of synaptic function and density (Attwell & Iadecola, 2002; Khatri & Man, 2013; Malonek & Grinvald, 1996; Rocher, Chapon, Blaizot, Baron, & Chavoix, 2003). FDG-PET studies suggest consistent and progressive reduction of CMRglc in AD of which extent and topography correlates with symptom severity (Mosconi, 2005). In advanced disease, it is suggested that hypometabolic regions are associated with parieto-temporal, posterior cingulate cortices (PCCs) and the frontal areas (Foster et al., 1984; Friedland et al., 1983; Minoshima et al., 1997). Since the 1980's, extensive research has focused on the metabolic disorder in AD brains and the findings above have been largely replicated, which renders this hypometabolism feature a reliable indicator for AD (Silverman et al., 2001).

Carrying the ApoE4 gene was first recognized as a risk factor for developing LOAD in 1993 with evidence that about 40% of AD patients have at least one ApoE4 allele. (Corder et al., 1993). Mean age of onset of AD is approximately 70 years for individuals

carrying two ApoE E4 alleles, 76 for one allele, and over 85 for the noncarriers, indicating an ApoE4 gene dose dependency (Farrer et al., 1997; Mosconi, Pupi, & De Leon, 2008). Individuals carrying an ApoE4 allele without dementia showed a mild but definite reduction in CMRglc in the same regions as clinically affected AD patients compared to noncarriers (L. Mosconi, S. De Santi, M. Brys, et al., 2008; Reiman et al., 2001; Reiman et al., 1996; Reiman et al., 2004; Small et al., 1995). This bioenergetic impairment is suggested to be progressive as well as closely related to the poor cognitive performance (Reiman et al., 2001; Small et al., 1995). Apart from the existing clues mentioned above, the exact molecular mechanism linking hypometabolism to ApoE and AD remain unclear.

The brain is one of the most ATP-consuming organs which is largely attributed to the high energy need of neuronal transmission and activity. Failure to maintain basal energy levels, such as under hypoglycemia or hypoxia, could induce synaptic loss and cognitive impairment within just a few minutes and render the brain vulnerable to energy deficit conditions (Fleck, Henze, Barrionuevo, & Palmer, 1993; Takata & Okada, 1995; Yamane, Yokono, & Okada, 2000). Rese arch has found that upon acute neuronal activity, uptake of glucose exceeds that consumed by oxidative metabolism, (Fox, Raichle, Mintun, & Dence, 1988) indicating glycolysis is widely used for neuronal function. The high level of utilization of glycolysis could be explained as below. Glutamate clearance from the synaptic cleft must be assisted by the Na/K-ATPase, a pump fueled by glycolysis, perhaps due to its faster delivery of ATP than oxidative phosphorylation (Campbell & Paul, 1992; Lipton & Robacker, 1983; Mercer & Dunham, 1981; Okamoto, Wang, Rounds, Chambers, & Jacobs, 2001; K. Wu, Aoki, Elste, Rogalski-Wilk, & Siekevitz, 1997). Failure to remove excessive glutamate will cause damage to neurons. The enzymatic machinery of glycolysis

also resides in the postsynaptic density (PSD) where it facilitates the regulation of AMPA receptor turnover via Na/K-ATPase, which is demonstrated to be correlated with Aβ-induced synaptic depression and the loss of dendritic spines (Hsieh et al., 2006; K. Wu et al., 1997; Zhang et al., 2009). Lujan et al. reported that inhibition of glycolysis, but not mitochondrial OxPhos, rapidly altered neurotransmission, resulting in highly variable, oscillating response. However, inhibition of mitochondrial-derived ATP had no significant effect on Ca^{2+} influx and did not alter the action potential nor the resultant EPSC (Lujan, Kushmerick, Banerjee, Dagda, & Renden, 2016). Additionally, synaptic vesicle refilling and fast axonal transport are reported to be preferentially fueled by glycolytic enzymes (Ikemoto, Bole, & Ueda, 2003; Ishida, Noda, & Ueda, 2009). In response to stress conditions, such as hypoxia, glycolytic enzymes are found to be aggregated near synaptic release sites (Zala et al., 2013). However, since energy metabolism research was traditionally focusing on oxidative phosphorylation for the great amount of ATP it generates, the exact function and mechanism involved with glycolysis in the brain has not been thoroughly understood.

Synaptic disruption and neuronal loss have been indicated in the brains of AD patients at a structural level, which is believed to have a close relationship with Aβ deposition (Querfurth & LaFerla, 2010; Serrano-Pozo, Frosch, Masliah, & Hyman, 2011; Skovronsky, Lee, & Trojanowski, 2006). Existing evidence suggest that Aβ is distributed uniquely in different brain areas and one of the striking features of these areas is their dependence on glycolysis (Buckner, Andrews-Hanna, & Schacter, 2008; Buckner et al., 2005; Vaishnavi et al., 2010; Vlassenko et al., 2010). Glycolysis is the first stage of cellular glycose metabolism. It breaks down glucose by converting one molecule of glucose to

ultimate substrates--- two molecules of pyruvate through 10 catalytic steps accompanied by a net production of 2 molecules of ATP and NADH + H^+ which then move on to the next step of cellular respiration. Three key rate-limiting enzymes have been identified in glycolysis --- hexokinase, phosphofructokinase-1 and pyruvate kinase. Hexokinase functions as the initial step of glycolysis and converts glucose to glucose-6-phosphate (G-6-P). G-6-P is a central molecule for the entry of glucose into both catabolic and anabolic pathways (Fig. 3). Free glucose can be provided by dephosphorylation of G-6-P to help maintain blood glucose levels. Isomerization of G-6-P to G-1-P is necessary to shunt glucose into glycogenesis. Oxidation of G-6-P to pentose phosphates by the pentose phosphate pathway leads to RNA/DNA and NADPH synthesis.

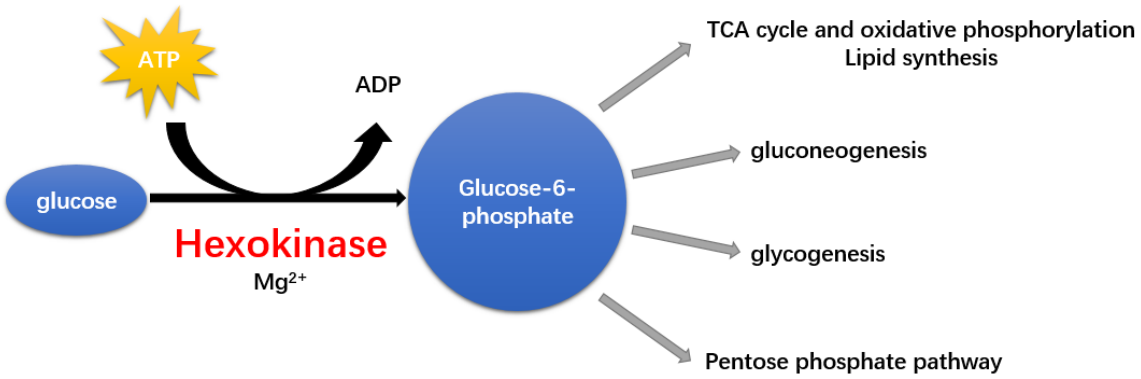


Figure 3. Central role of glucose-6-phosphate as an entry point into both oxidative and anabolic pathways of glucose utilization.

All these reactions require a proper function of hexokinase to form G-6-P. Hexokinase exists in four isoforms with the most common types being HKI and HKII. In the CNS, HKI is the major isoform, often indicated as "the brain HK" (Lowry & Passonneau, 1964; Purich

& Fromm, 1971). Metabolic measurements suggest that HKI exclusively promotes glycolysis, thus is more related to catabolic metabolism. On the contrary, HKII, of which expression is relatively less in the brain, is more associated with anabolic use. Additionally, HKII appears to function in a more complex manner depending on various subcellular localizations; it can enhance glycolysis when bound to mitochondria, but facilitate glycogen synthesis when located in the cytosol (John, Weiss, & Ribalet, 2011). In the CNS, HK appears to be highly involved in regulating neuroprotective mechanism. Research suggest that HK promotes glycolysis in cortical neurons in a Wnt dependent manner, the Wnt signaling pathway crucially regulates synaptic cleft structure and neuroprotection against injury. Notably, this Wnt-mediated neuroprotection is reported to be independent of GLUT3, glucose-6-dehydrogenase or the pentose phosphate pathway (Cisternas, Salazar, Silva-Alvarez, Barros, & Inestrosa, 2016). It is reported that HKII also functions as an enhancer for neuronal survival acting downstream of glycogen synthase kinase-3 (GSK-3), which has been long considered as a critical factor in regulating neuronal cell survival and death (Gimenez-Cassina, Lim, Cerrato, Palomo, & Diaz-Nido, 2009). Collectively, these findings suggest that HK may function as a neuroprotective promoter against neurodegenerative diseases which are characterized by energy metabolism disorders, such as AD.

In the present study, we applied both stably human ApoE-expressing Neuro-2a cell lines as w ell as a transiently transfected neuronal cell model to confirm the role of ApoE in regulating glycolytic function via HK. We found that hApoE2-expressing cells exhibited a higher level and activity of both HK isoforms as well as glycolytic parameters compared to hApoE4-expressing cells. In addition, metabolic activity and morphological property

were also differentially impacted by the three ApoE isoforms in line with regulation of HK. Intriguingly, these distinctive regulations were independent of glucose transporter 4 and apoptosis regulator BAX. Taken together, our results provide evidence to support that ApoE2-mediated neuroprotective effects are exerted through the up-regulation of glycolysis via HK, which could potentially serve as a mechanistic rationale underlying the differential risk of the three ApoE isoforms in the onset of AD.

Materials and methods

Plasmids

Human ApoE2, ApoE3 and ApoE4 cDNA expressed in the mammalian vector pCMV6 with C-terminal Myc-DDK Tag were obtained as previously described.(Long Wu, 2017). The empty mammalian pCMV6 vector was used as control for all the experiments. Amplification was performed by transforming the plasmids into DH5α competent cells (Invitrogen Life Technologies, CA, USA). The purification of plasmids was conducted by using Plasmid Midi Kit as described by the manufacturer (Qiagen, Hilden, Germany).

Generation of Neuro-2a Cells Stably Expressing Human ApoE isoforms

Mouse neuroblastoma cells stably expressing human ApoE isoforms were obtained as described previously (Long Wu, 2017). Briefly, optimal dose of antibiotics (G418) was determined by titration and used for a kill curve on Neuro-2a cells. 2ug of hApoE2/3/4-pCMV plasmid were transfected using Lipofectamine 3000 according to the manufacturer's instructions. After 48 hours the medium was replaced and supplemented with G418. Cells were maintained for 10 days, then dissociated from the plates and subjected to limited dilution on 96-well plates. Half the medium was changed every 3-4 days and after 10-14 days, the cells were transferred for further expansion and study.

Immunoblotting

Cells were rinsed 2-3 times with cold phosphate-buffered saline (pH 7.4, Thermo Fisher Scientific, MA, USA) and lysed in neuronal protein extraction reagent (NPER) or RIPA lysis containing protease and phosphatase inhibitors (Thermo Fisher Scientific, MA,

USA) for 15-30 minutes on ice. Whole cell lysate was then centrifuged at 4 °C for 10mins at 10,000 x g. The supernatant was collected and protein concentration of the cell extracts was determined by the BCA protein assay kit (Thermo Fisher Scientific, MA, USA). After normalization of the protein concentration, samples were diluted in Laemmli sample buffer (Bio-Rad, CA, USA) with 2-mercaptoethanol (Bio-Rad, CA, USA) and boiled at 95 °C for 5mins. Equal amounts of total protein was loaded in each lane and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto 0.2 μm pore-sized PVDF membranes (Bio-Rad, CA, USA). The membrane was blocked with 5% non-fat milk in TBS (100ml 10×TBS (200mM Tris, 1.5M NaCl), 900ml ddH_2O, pH 7.6) for 1 hour at room temperature, followed by incubation with primary antibody at 4 °C overnight. The membranes were then washed with TBST (100ml 10×TBS (200mM Tris, 1.5M NaCl), 10ml 10% Tween-20, 890ml ddH_2O, pH 7.6) for 4 times, 5 mins for each, followed by hybridization with the horseradish peroxidase (HRP)-conjugated secondary antibody. After another 4 washes of 5 minutes each, the bands on the membrane were detected by C-Digit Blot Scanner (LI-COR, Lincoln, NE) after applying the enhanced chemiluminescence (ECL) reagent (Bio-Rad, CA, USA). The western blots were scanned and quantification was obtained using the Image Studio Version 4.0 imaging digitizing software standardized by the internal loading control protein. The following antibodies were applied: goat anti-Apolipoprotein E (1:4000, EMD Millipore), rabbit anti-hexokinase I (1:2000, Cell Signaling Technology, Danvers, MA), rabbit anti-hexokinase II (1:1500, Cell Signaling Technology), mouse anti-GAPDH (1:6000, Santa Cruz Biotechnology, Inc), rabbit anti-BAX (1:1000, Cell Signaling Technology), rabbit anti-GLUT-4 (1:1000, EMD Millipore), rabbit anti-NeuN (1:1000, Cell Signaling Technology),

rabbit anti-synaptophysin (1:1000, Cell Signaling Technology), rabbit anti-PSD95 (1:1000, Cell Signaling Technology), rabbit anti-β-actin (1:5000, Thermo Fisher Scientific, MA, USA), and mouse anti-β-tubulin (1:5000, Thermo Fisher Scientific, MA, USA).

Hexokinase activity assay

Cell lysate was prepared by lysis in cold NPER or RIPA buffer containing protease and phosphatase inhibitors then centrifuged at 1,500g for 5mins at 4°C. The supernatant was collected and hexokinase activity was measured as the total glucose phosphorylating capacity of the lysate based upon the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase. Results were measured spectrophotometrically by monitoring the increase in absorbance at 340 nm every 1 minute for 30minutes under 37°C. The initial linear portion of curve was used to determine ΔA/min for further calculation. Briefly, hexokinase assay solution was prepared with 13.3 mM MgCl2, 0.112 M glucose, 0.55 mM adenosine 5' triphosphate, 0.227 mM NAD^+, and 1 IU/mL glucose-6-phosphate dehydrogenase in 0.05 M Tris-HCl buffer, pH 8.0 as described previously with minor modification (Ding, Yao, Rettberg, Chen, & Brinton, 2013). After 6-8 minutes of incubation at room temperature to reach equilibrium, 15-20 µl cell extracts was added into 150 µl assay solution to initiate the reaction. Diluted hexokinase solution served as the positive control, while assay solution without cell extracts was applied as the negative control. Results were normalized to total cellular protein content using the BCA standard assay.

Glycolytic stress test

Extracellular flux (XF) analysis (Agilent Technologies) was used to evaluate the glycolytic stress. Basal glycolysis and glycolytic capacity were measured by assessing the extracellular acidification rate (ECAR). Cells expressing human ApoE isoforms were seeded at a density of 8,000-10,000 cells/well on Seahorse XF96 culture plates in complete growth medium the day before the experiment. On the experiment day, glycolytic stress test assay medium was prepared as XF base medium (Agilent Technologies) containing 2 mM glutamine, pH 7.4. The assay was initiated by replacing the complete growth medium with pre-warmed assay medium and incubating the cell culture plate at 37°C without CO2 at least 1hour prior to the first rate measurement. Glycolytic parameters of hApoE2/3/4-expressing cells were then measured using the Seahorse XF Analyzer according to manufacturer's instruction following successive injections of 10 mM glucose, 2 μM oligomycin, and 50 mM 2-Deoxy-D-glucose to each well. Basal glycolysis was indicated after the initial injection of glucose while the glycolytic capacity was assessed after the injection of oligomycin, which inhibits mitochondrial respiration. The last injection of 2DG, a glucose analogue, functions as a competitive inhibitor of the production of glucose-6-phosphate, thereby the glycolysis test was terminated. Cell culture plates were saved after the assay and used for the normalization of the results by assessing the protein concentration in each well. Data were analyzed by the report generator for XF glycolysis stress tests.

LIVE/DEAD Cell assay

Stable human ApoE2/3/4-expressing cells were seeded on ibiTreat #1.5 polymer

coverslip μ-Slide (Ibidi, Martinsried, Germany) with complete growth medium on day 0. The medium was replaced with fresh medium containing G418 every two days. On day 4, the culture medium was removed carefully and replaced with the LIVE/DEAD cell assay reagents at the working concentration according to the manufacturer's instruction together with Hoechst dye to stain nuclei. The LIVE/DEAD cell assay reagents are not fluorescent unless they interact with cells. After applying the LIVE/DEAD cell assay reagents to the cells, live cells characterized by the ubiquitous presence of intracellular esterase will convert the non-fluorescent cell-permeable calcein AM (495/515 nm) into calcein, which will produce a green fluorescence, whereas the red component is cell membrane impermeable, thus only can be generated by dying/dead cells upon binding to DNA. Images were obtained under the Leica DMI4000 B inverted microscope with FITC (495-519 nm) and Texas Red (595-605 nm) filters using microscope software of Leica Microsystems. 10x (HCX PL APO 10x/0.40 NA 2.20 WD) magnification was used. 10-15 images of interest were obtained for each isoform in each passage with similar cell density. Hoechst staining and dead cells were used as control.

Morphological change analysis

Stable human ApoE2/3/4-expressing cells were seeded in 35mm glass-bottom poly-D-lysine pre-coated culture dishes (MatTek corporation, MA, USA) at a cell density of 3×10^4/ml on day 0. Cells were maintained for 4 days and the medium replaced with fresh complete culture medium containing G418 every 2 days without phenol red (Thermo Fisher Scientific, MA, USA). All three stable cell lines were treated with the same condition. Phase contrast images were acquired using an Olympus IX81 inverted

epifluorescence microscope equipped with a cage incubator for maintaining precise temperature, humidity and CO2 control. Slidebook Software Version 6.0 was used for microscope control, image acquisition, image processing, and data analysis (Intelligent Imaging Innovations, Inc., Denver, CO). Images were acquired using a 40× phase contrast magnification, 40× LWD (N.A.=0.6, W.D.=0-2) and a Zyla 4.2 PLUS sCMOS camera with a 1.0x Infinity Space Adaptor (Andor Technology Ltd., Belfast, UK) on day 4 under 37°C and 5% CO2.

Metabolic activity assay

A cell permeable resazurin-based solution, PrestoBlue® (Invitrogen Life Technologies, CA, USA), was applied as a cell viability indicator by using the reducing power of living cells for quantitative measurement. 10 µl of PrestoBlue reagent was directly added to cells in 90 µl of culture medium in a 96-well plate. The plate was incubated at 37 °C, for 1h and fluorescence was read at Ex/Em 528/20,600/40 nm according to manufacturer's instruction using a plate reader (BioTek). Wells containing only cell culture media (no cells) were used as background control wells. Backgrounds values were subtracted from all readings. Results were normalized to protein content of each well. Higher fluorescence units indicate greater metabolic activity.

Cell culture

N2a cells stably expressing human ApoE2/3/4

Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA,

USA) containing 600 μg/mL Geneticin (G418, Thermo Fisher Scientific, MA, USA) in a humidified incubator under an atmosphere of 5% CO2 at 37 °C. The cells were sub-cultured every 3-4 days or when the confluency reached about 90%. New vials of frozen stock cells were recovered when the cell passages exceeded 20.

RA-induced neuronal differentiation

The Neuro-2a cell line, the neuroblast cell type from mouse brain, is often stimulated with retinoic acid to differentiate into neuron-like cells to model the response of neurons (Tremblay et al., 2010; P. Y. Wu et al., 2009). To study this neuron model, Neuro-2a cells obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were seeded on 6-well plates at a cell density of 1×10^5/mL in complete culture medium for 24 hours. The medium was then changed to DMEM supplemented with 2% FBS containing 20 μM all-*trans*-RA (Sigma-Aldrich, Inc, St. Louis, MO). The medium was replaced with fresh medium containing RA every 2 days. After 4 days, neuron-like differentiated Neuro-2a cells were identified by neuronal morphological characterizations using phase contrast imaging as well as several neuronal biomarkers, NeuN, synaptophysin and PSD95 using western blots. Differentiated neurons were then subjected to treatments and further studies**.**

Transfection of human ApoE isoforms in neurons

Transient transfection of human ApoE isoforms was conducted 96 hours after RA-induced differentiation of the Neuro-2a cells using Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen Life Technologies, CA, USA) according to manufacturer's instruction. 250μl of the transfection reagent mixture and 3μg plasmid DNA was added to

the cells in triplicate wells. The transfection mixture-containing medium without FBS was replaced by medium containing RA after 6 hours. Cells were maintained for another 96 hours allowing the protein to be expressed, then subjected to further studies. Transfection efficiency was determined by protein expression using western blot.

Lactate production assay

Lactate fluorometric assay kit (K607-100) (BioVision Inc) was used to determine lactate levels according to the manufacturer's instructions. Briefly, differentiated N2a cells transfected with ApoE isoforms were incubated in DMEM without FBS or glucose for 1 hour. The medium was refreshed and cells were incubated in 37°C incubator without CO2 for another 1 hour. The assay was started by adding 10 mM glucose to each well of transfected cells followed by incubation in 37°C, 5% CO2 for 10 minutes. 10 µL medium was collected from each sample and incubated with reaction mix for 30 minutes at room temperature. The fluorescence was detected at ex/em 540/35,600/40 nm using a plate reader (BioTek). Lactate levels were normalized to protein content in each sample.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used to conduct statistical analyses. Data represent the group mean ± standard deviation. For statistical comparisons, quantitative data were analyzed by Student’s t-test or one-way analysis of variance (An et al.) with Tukey's Post hoc Test. P value lower than 0.05 was considered as statistically significant.

Results

1. Generation and validation of human ApoE2, ApoE3, and ApoE4-expressing stable cell lines.

Neuro-2a (N2a) cells stably expressing human ApoE2, ApoE3, or ApoE4 were generated as described (Long Wu, 2017). To screen the ApoE expression level, western blot was performed for all 3 isoforms in the established stable cell lines. We aimed at identifying clones having similar ApoE expression for each isoform. A representative western blot of screening is provided in figure 4. Arrows indicate the targeted cell lines that showed comparable ApoE expression and were thus chosen as the final set for our following studies (Fig.4a). Considered as the starting point of a time-course study, ApoE expression levels of 3 isoforms in passage 5 were reconfirmed using immunoblotting (Fig.4b). Data represent the fold change (FC) relative to protein expression levels compared to ApoE3 no.7 group.

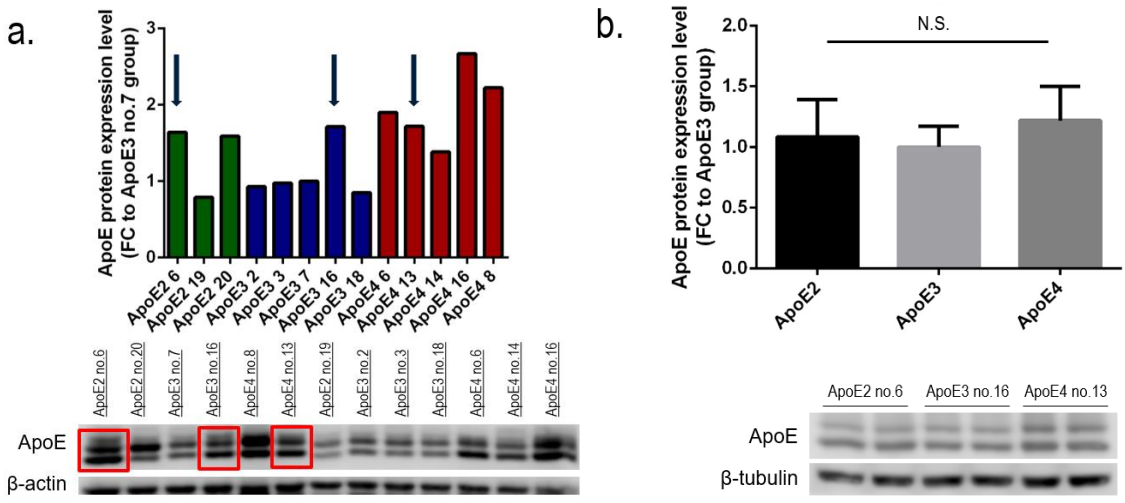


Figure 4. Generation and validation of human ApoE2/3/4-expressing stable cell lines. a) Examination of ApoE protein expression levels in hApoE-expressing stable lines / clones at passage 1 using western blot. All lines / clones were normalized to ApoE3 no.7. n=1 per group. Arrows indicate a potential stable cell line of each isoform that has a similar ApoE expression. b) Comparison of ApoE protein expression levels in the chosen ApoE2/3/4 stable cell lines at passage 5. No significant difference in ApoE expression validated the set working as a baseline for future study. Data were normalized to an internal loading control (β-actin or β-tubulin). Results were normalized to the ApoE3 group and analyzed using one-way ANOVA with Tukey's Post hoc Test. Data represent the group mean± SD. n=7 per group.

2. Hexokinase expression remained relatively stable in hApoE2 and ApoE3-expressing cells but exhibited a gradual decrease in hApoE4-expressing cells with increasing passages.

Using the chosen hApoE2/3/4-expressing stable cell lines, we first examined how ApoE isoforms individually impacted hexokinase in a time-dependent manner. Cells were thawed from liquid nitrogen at passage 5 and allowed 2 passages for them to recover. Cell lysate of hApoE2/3/4-expressing cells were harvested at every other passage beginning at P7. Both isoforms of hexokinase were probed using western blot (Fig.5). The data showed a gradual decrease in the expression of HK1 and HK2 in hApoE4-expressing cells with increasing passages starting at P11. In contrast, the expression of HK1 and HK2 remained relatively unchanged from P7 to P15 in hApoE2 and ApoE/3-expressing cells. These results indicate that hexokinase is differentially regulated by human ApoE isoforms and it appears to be chronically and negatively impacted by ApoE4.

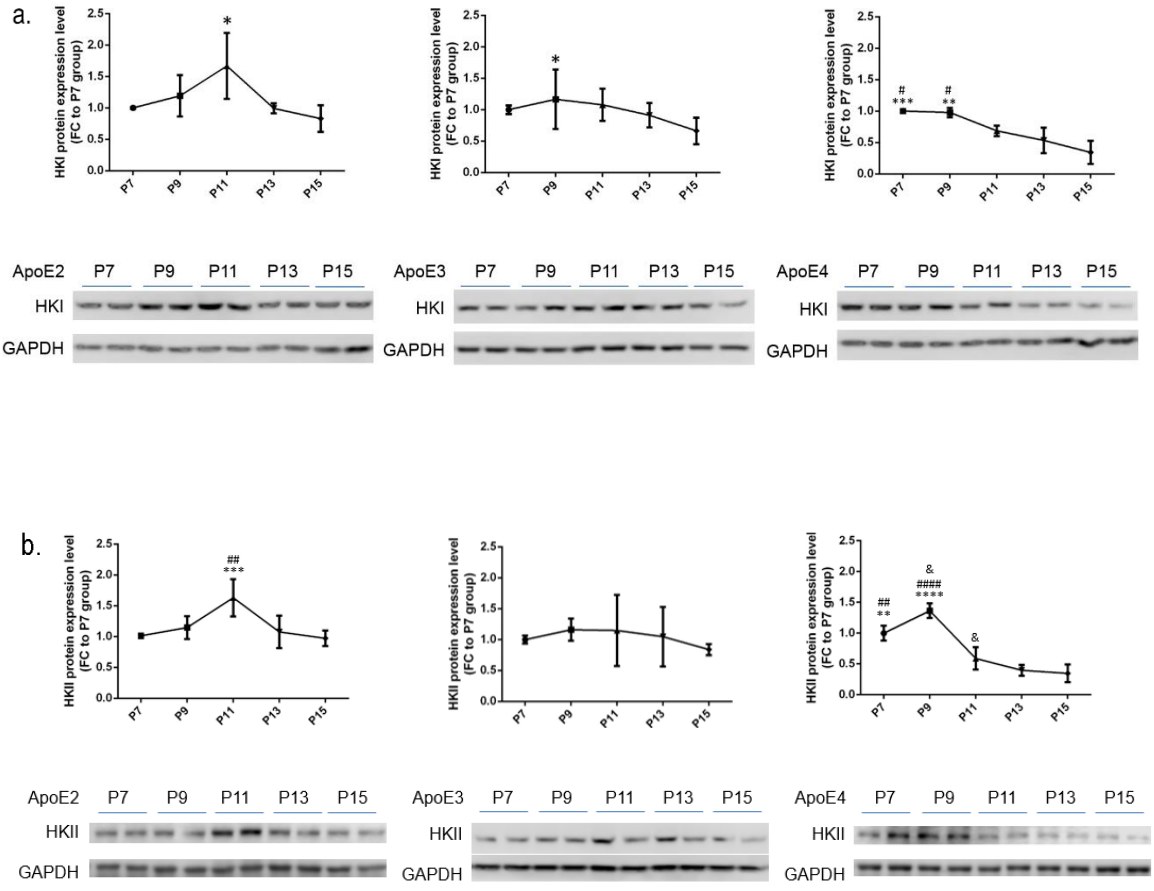


Figure 5. Hexokinase expression remained relatively stable in hApoE2 and ApoE3-expressing cells, however, a gradual decrease with increasing passages occurred in hApoE4-expressing cells, indicating a chronic and negative impact by ApoE4. Representative western blots (a-b) of hexokinase I & II protein expression level in hApoe2/3/4-expressing cells of every other passage. A gradual and significant decrease was observed in hApoE4-expressing stable cell line compared with hApoE2/3-expressing cells. Data were normalized to an internal loading control (GAPDH). Results were normalized to P7 group and compared using one-way ANOVA with Tukey's Post hoc Test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs P15 group, #p<0.05 ##P<0.01 ####P<0.0001 vs P13 group, &P<0.05 vs P7. Data represent the group mean± SD. n=3-6 per group.

3. Human ApoE and GLUT4 expression were unchanged with increasing passages in all 3 ApoE cell lines.

Human ApoE expression level of each stable cell line was also examined every other passage. As shown in Fig.6, protein levels of ApoE remained comparable, further validating the 3 stable cell lines. Additionally, based on our previous study (Long Wu, 2017), we also tested the GLUT4 protein level of these 3 stable cell lines using western blot. However, no statistically significant difference was found between the passages. These data indicate that our human ApoE2/3/4-expressing stable cell lines remained valid within all studied passages and the regulation of hexokinase by ApoE isoforms is independent of changes in the expression of the glucose transporter, GLUT4.

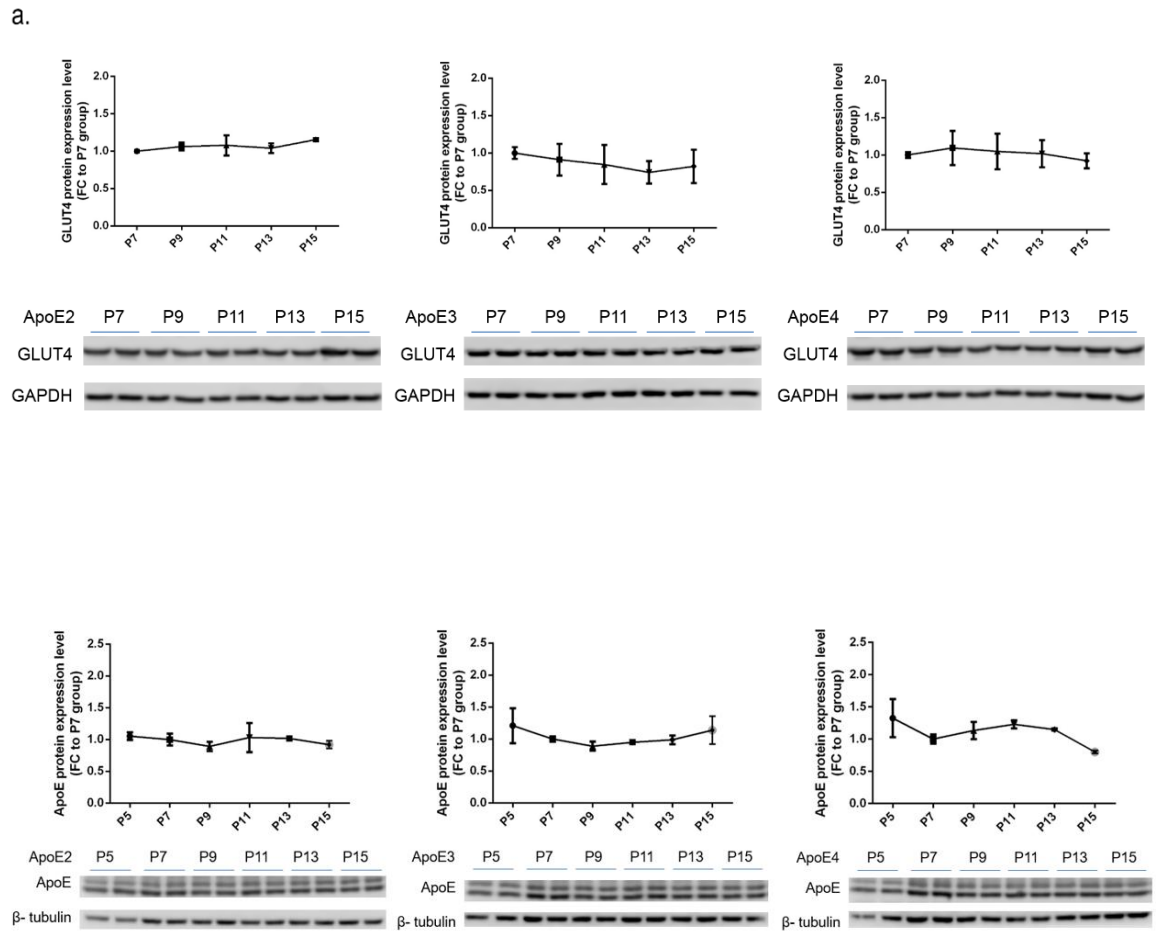


Figure 6. GLUT4 and ApoE protein expression remained similar within passages of three ApoE stable cell lines. Expression of glucose transporter type 4 and ApoE of all passages studied were assessed via western blot. No significant difference was observed. Data were normalized to an internal loading control (β-tubulin or GAPDH). Results were normalized to P5 or P7 group and compared using one-way ANOVA with Tukey's Post hoc Test. Data represent the group mean± SD. n=2-6 per group.

4. hApoE2 upregulated whereas hApoE4 downregulated the expression of both isoforms and the activity of hexokinase.

Given that a significant difference in HK isoforms was revealed around passage 11, we assessed protein expression and activity of hexokinase of three ApoE stable cell lines at P9, P11 and P13. In all of three passages, compared to the ApoE3 group, hApoE2-expressing cells exhibited a higher protein expression of both isoforms of hexokinase whereas hApoE4-expressing cells showed a remarkably lower expression (Fig.7a). In line with the hexokinase protein expression data, hApoE2-expressing cells exhibited a notably higher level of enzyme activity whereas hApoE4-expressing cells demonstrated a significantly decreased activity compared to ApoE3 group (Fig.7b). These findings suggest that hApoE2 upregulates whereas hApoE4 downregulates the expression of both isoforms and the activity of hexokinase.

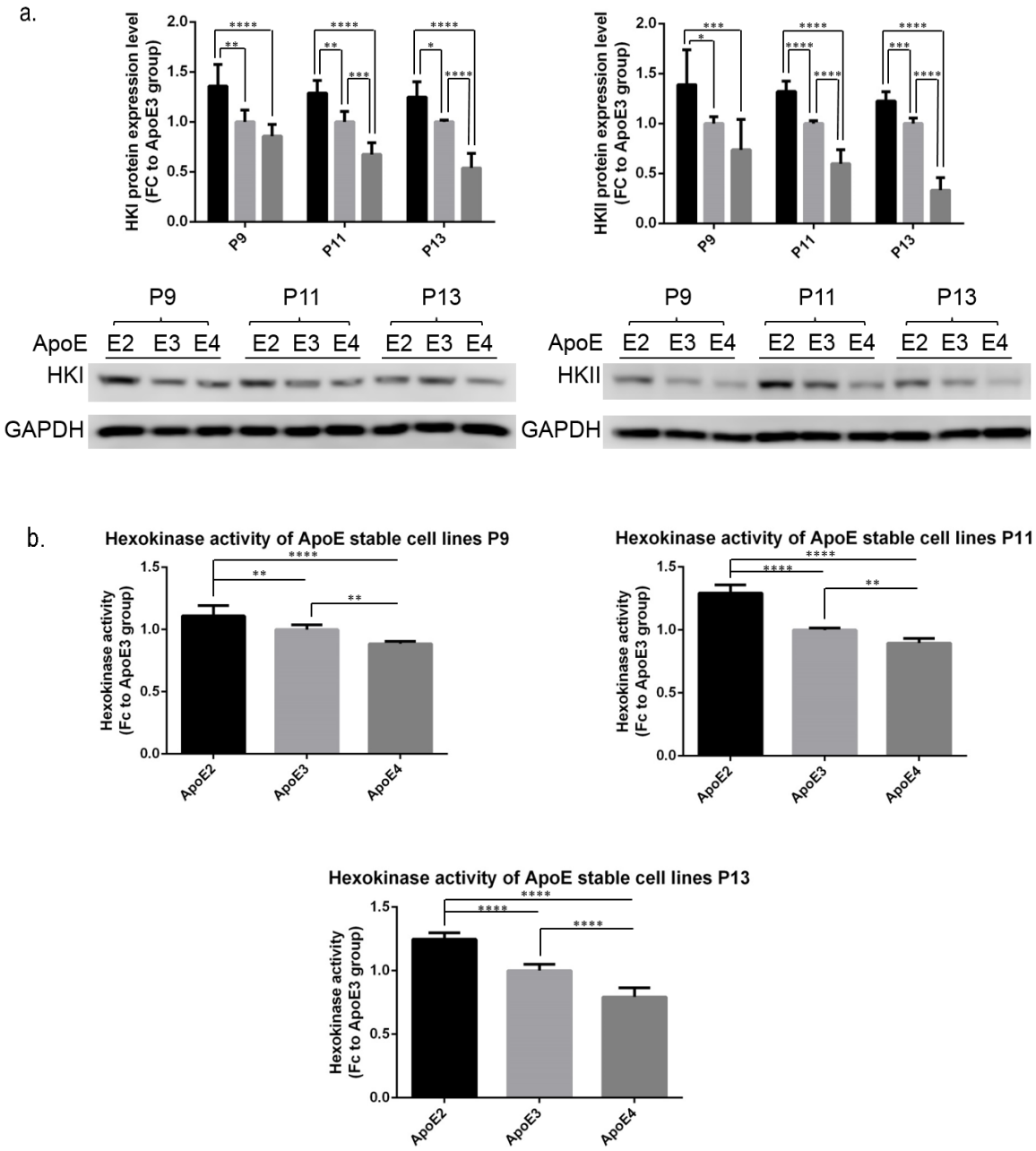
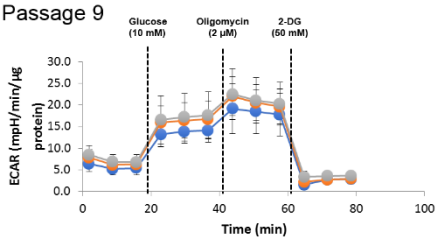


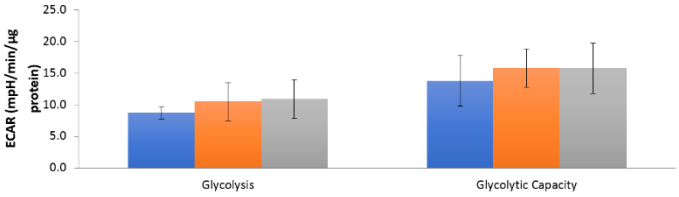
Figure 7. When compared to hApoE3, hApoE2 upregulates whereas hApoE4 downregulates the expression of both isoforms and the activity of hexokinase. At P9, P11 and P13, Hexokinase I&II (a) protein expression levels were assessed using immunoblotting analyses. Enzyme activity (b) was examined using hexokinase activity assay. Data indicate hApoE2-expressing cells displayed the highest while ApoE4 cells exhibited the lowest enzyme activity and protein expression level of both hexokinase I & II. Data were normalized to an internal loading control (GAPDH). Results were normalized to ApoE3 group in each passage and compared using one-way ANOVA with Tukey's Post hoc Test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data represent the group mean± SD. n=5-10 per group.

5. hApoE2-expressing cells exhibited enhanced glycolytic activity when compared to ApoE4-expressing cells.

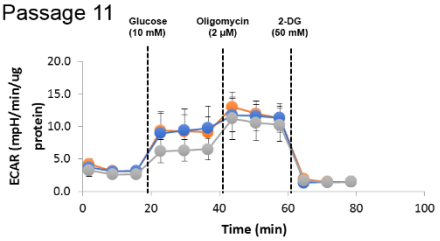
Since hexokinase is the first rate-limiting enzyme in intracellular glucose metabolism and functions as the "pacemaker" in glycolysis, further assessment of glycolytic function was conducted on the three ApoE stable cell lines using a Seahorse glycolysis stress test kit. In this assay, 10 mM glucose, 2 μM oligomycin and 50 mM 2-Deoxy-D-glucose were sequentially injected into each well while the measurement of extracellular acidification rate was made. Glycolytic rate was measured after the supplement of glucose. Oligomycin, an inhibitor of ATP synthase in the ETC, drives glycolysis to its maximum capacity. Finally, 2-DG terminated glycolysis by competitively inhibiting hexokinase. The non-glycolytic ECAR of cells was then measured. As shown in figure 8, starting form passage 11, the glycolysis rate of hApoE4-expressing cells was significantly lower compared to the ApoE3 group. Meanwhile, at P11 and P15, the glycolysis rate of hApoE2-expressing cells was significantly higher than that of the hApoE3-expressing cells. Glycolytic capacity induced by addition of oligomycin has had a lower magnitude in hApoE4-expressing cells compared to the ApoE3 group, while hApoE2-expressing cells had a greater increase than the ApoE4 group at passage 15. These data indicate that hApoE2-expressing cells exhibited enhanced glycolytic activity whereas hApoE4-expressing cells are relatively deficient.

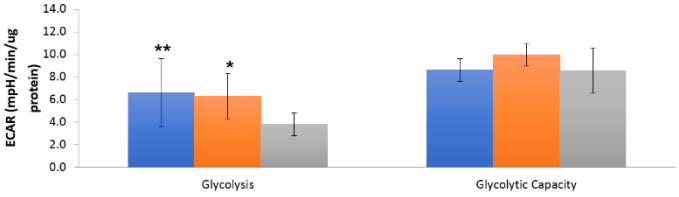
Passage 9





Passage 11





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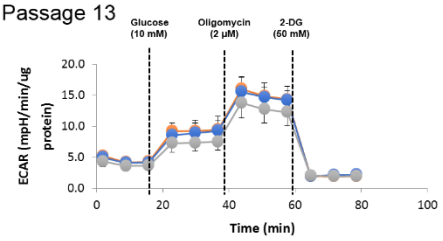
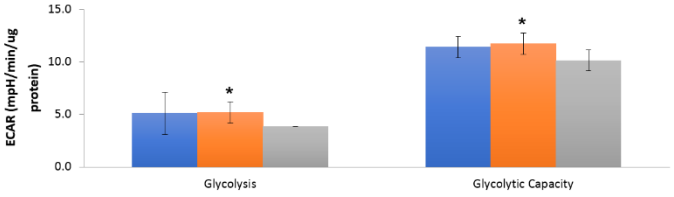
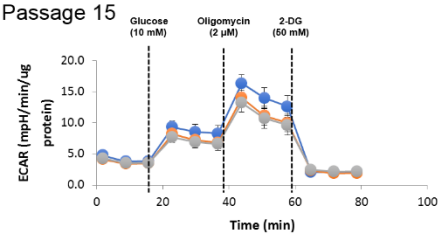




Passage 15



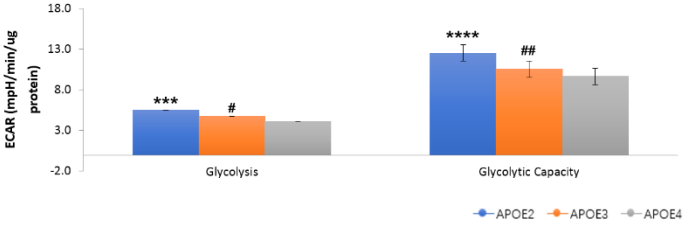


Figure 8. hApoE2-expressing cells exhibited enhanced glycolytic activity when compared to ApoE4-expressing cells. Glycolytic function of hApoE2/3/4-expressing cells were analyzed using the Seahorse XF96 Extracellular Flux Analyzer following the sequential injections of 10 mM glucose, 2 μM oligomycin and 50 mM 2-Deoxy-D-glucose. Results of ECAR, basal glycolysis and glycolytic capacity were shown above. Data indicate that hApoE4-expressing cells demonstrated a relative deficit in all parameters of glycolysis when compared to hApoE2/3-expressing cells. Results were compared using one-way ANOVA with Tukey's Post hoc Test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs ApoE4 group, #p<0.05, ##p<0.01, vs ApoE2 group. Data represent the group mean ± SD. n=10-16 per group.

6. hApoE2-expressing cells maintained the most robust health status as evidenced by both metabolic activity and morphological analysis.

Given that human ApoE isoforms distinctively modulate expression and activity of hexokinase, further affecting glycolytic function, we next investigated whether this regulation also influenced overall cell metabolic activity and health status. A cell permeable resazurin-based solution, PrestoBlue®, was used as a cell viability indicator by using the reducing power of living cells for quantitative measurement. Under non-stressed conditions, hApoE2/3/4-expressing cells displayed differential metabolic activity at passage 9, 11 and 13 indicated by the fluorescence units after normalization for protein contents. Compared to the ApoE3 group, ApoE2 cells had a higher metabolic activity while ApoE4 cells exhibited a lower level of metabolic activity (Fig.9a). Additionally, overall cell health status indicated by morphological integrity of the three stable cell lines was further examined. Morphology by phase contrast imaging suggested that ApoE2 cells presented a healthier morphological monolayer and regular cell shape whereas ApoE4 cells were more likely to aggregate into massive clumps (Fig.9b). Collectively, these findings showed that hApoE2-expressing cells demonstrate an elevated level of metabolic activity which correlated to an overall better cell heath status.

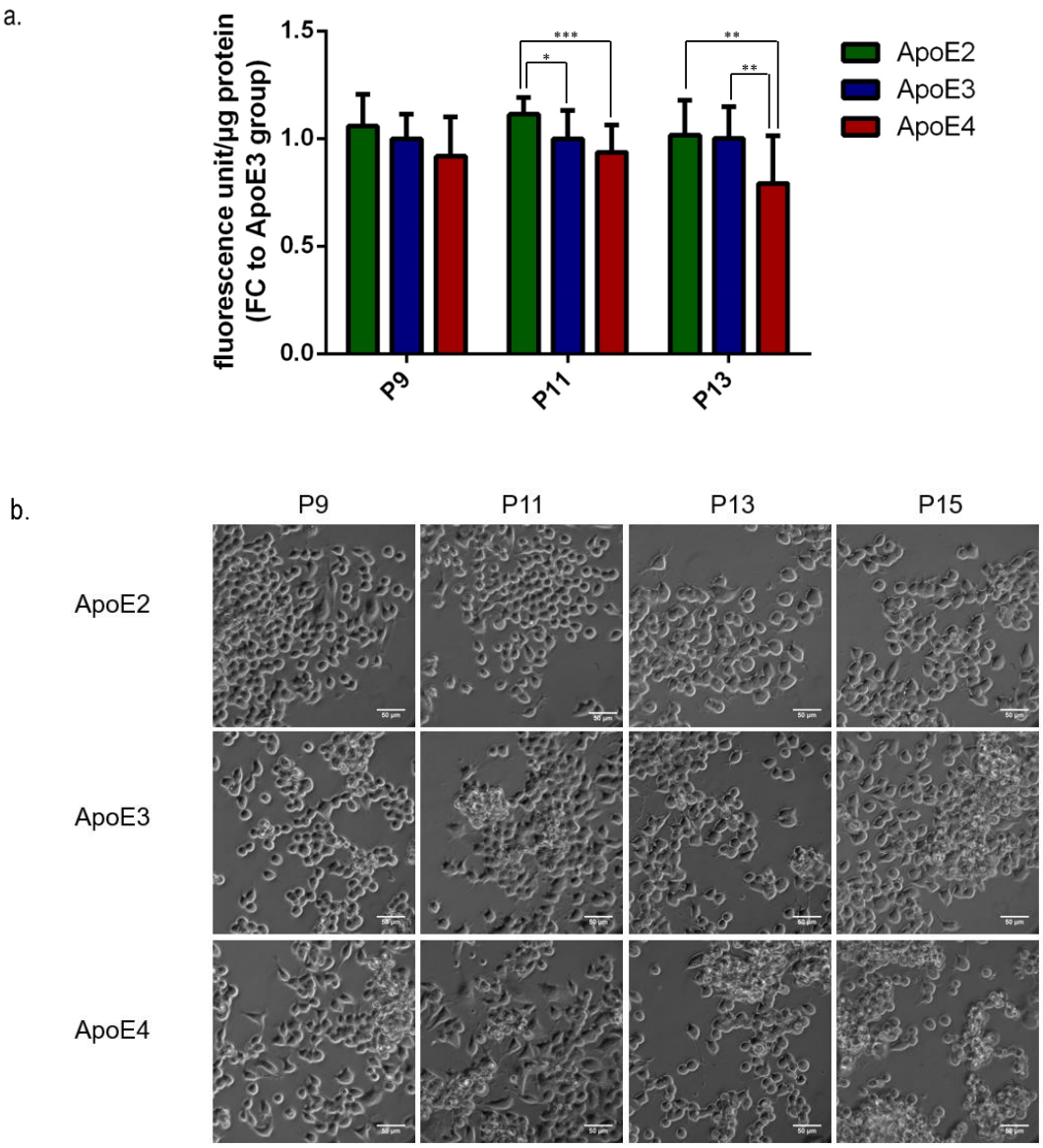
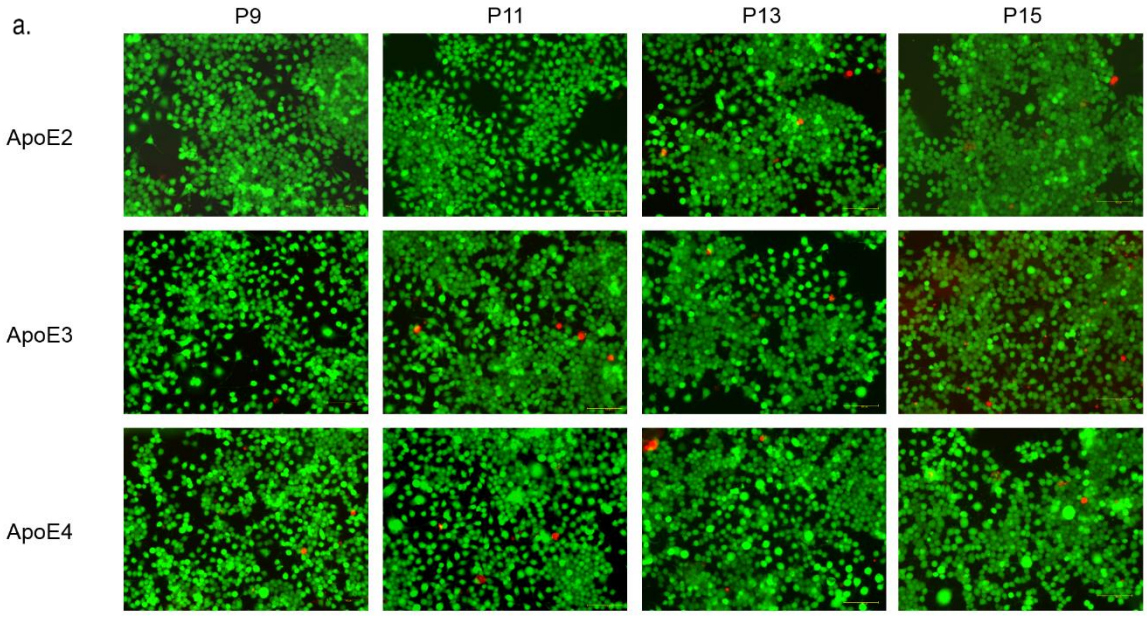
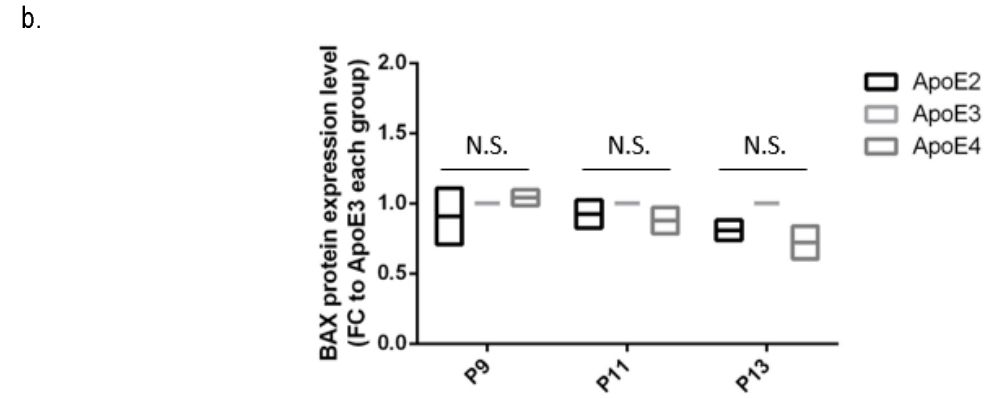


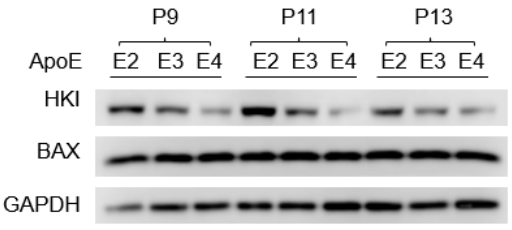
Figure 9. hApoE2-expressing cells maintained the most robust health status as evidenced by both metabolic activity and morphological phenotype. (a) ApoE2/3/4-expressing cells were seeded on 96-well plate for 4 days followed by PrestoBlue™ metabolic activity analysis. Results were normalized by protein content in each well. This finding suggest that metabolic activity was the most robust in hApoE2-expressing cells whereas hApoE4-expressing cell was the most deficient. (b) Representative phase contrast images were obtained at day 4 after seeding at corresponding passages under the same conditions. Consistent with metabolic activity data, morphological characteristics analysis indicate that ApoE2 cells presented a healthier morphological behavior with monolayer and regular shape of cells whereas ApoE4 cells were more likely to aggregate into massive clumps. Scale bars, 50 µm.

7. Metabolic deficiency and morphological abnormality associated with ApoE4 is independent of apoptotic process.

We next examined whether apoptosis was involved in this modulation of cell health status and metabolic activity by ApoE isoforms. A LIVE/DEAD Cell assay was performed and no significant death was observed among the ApoE2/3/4-expressing cells within all passages studied (Fig.10a). The apoptosis regulator BAX, also known as bcl-2-like protein 4, is an apoptosis activator involved in a wide variety of bio-cellular activities. It is reported that upon the initiation of apoptotic signaling, BAX will undergo a conformational change which will lead to apoptosis via increasing the opening of the mitochondrial voltage-dependent anion channel (VDAC) (Gross, Jockel, Wei, & Korsmeyer, 1998; Hsu, Wolter, & Youle, 1997; Nechushtan, Smith, Hsu, & Youle, 1999; Shi et al., 2003; Wolter et al., 1997). Western blot was performed assessing the protein expression level of BAX in hApoE2/3/4-expressing cells (Fig.10b). No significant difference was observed in the three stable cell lines at passage 9, 11 and 13. Notably, both isoforms of hexokinase expression were remarkably regulated by ApoE isoforms. These results indicate that metabolic deficiency and morphological abnormality observed in hApoE4-expresing cells is independent of apoptosis and the data highlight the specific role on the regulation of hexokinase conferred by ApoE isoforms.


a.
P9
P11
P13
P15
ApoE2
ApoE3
ApoE4


b.
BAX protein expression level
(FC to ApoE3 each group)
2.0
1.5
1.0
0.5
0.0
N.S.
N.S.
N.S.
P9
P11
P13
ApoE2
ApoE3
ApoE4


P9
P11
P13
ApoE
E2 E3 E4
E2 E3 E4
E2 E3 E4
HKI
BAX
GAPDH

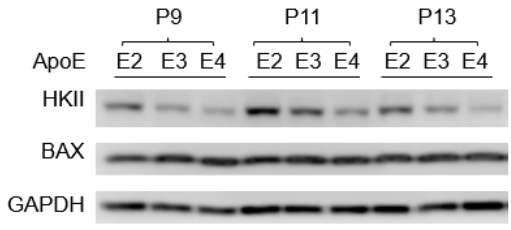

P9
P11
P13
ApoE
E2 E3 E4
E2 E3 E4
E2 E3 E4
HKII
BAX
GAPDH

Figure 10. Metabolic deficiency correlated to overall cell health status by ApoE4 is independent of apoptotic process. (a) No significant death was observed evidenced by both LIVE/DEAD cell fluorescent images and (b) western blot of apoptosis regulator BAX indicating that the negative impact by ApoE4 is independent of apoptotic process highlighting the importance of glycolysis in energy metabolism regulated by ApoE isoforms. Scale bars, 100 μm. Image regions of interest were determined as those having similar cell density at about 80%-90% confluency. Data were normalized to an internal loading control (GAPDH). Immunoblotting results were normalized to ApoE3 group in each passage and compared using one-way ANOVA with Tukey's Post hoc Test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Data represent the group mean± SD. n=2 per group.

8. Morphological and immunocytochemical characterization of 96h RA-induced differentiated Neuro-2a cells.

Previous studies reported that Neuro-2a cells can be stimulated by retinoic acid (RA) to differentiate into neuron-like cells (Tremblay et al., 2010; P. Y. Wu et al., 2009). To study ApoE effects on neurons, we differentiated Neuro-2a cells for 96h in DMEM containing 20 μM RA, followed by the characterization of the differentiated cells for their morphological and biochemical phenotypes. Morphological analysis by phase contrast imaging confirmed the elaboration of neurites consistent with the development of neuron-like features (Fig.11a). Additionally, several neuronal biomarkers were assessed by immunoblotting. NeuN has been universally considered as a neuron-specific marker for differentiation (Gusel'nikova & Korzhevskiy, 2015). Synaptophysin, a synaptic vesicle glycoprotein ubiquitously presents at the pre-synapses, regulates vesicle formation and endocytosis in neurons (Wiedenmann & Franke, 1985). PSD-95 (postsyna ptic density protein 95), is associated with receptors and the cytoskeleton which plays an important role in synaptic plasticity and regulating synaptic maturation (Funke, Dakoji, & Bredt, 2005; Gilman et al., 2011; Kim & Sheng, 2004; Meyer, Bonhoeffer, & Scheuss, 2014). Results in (Fig.11b) revealed a significant increase in NeuN, synaptophysin and PSD95 compared

to non-differentiated cells. Together with the morphological analysis, these data confirmed the neuronal phenotypes of 96h RA-induced differentiated N2a cells, providing an in vitro neuronal model for our subsequent examination of ApoE effects on hexokinase and glycolysis in neurons.

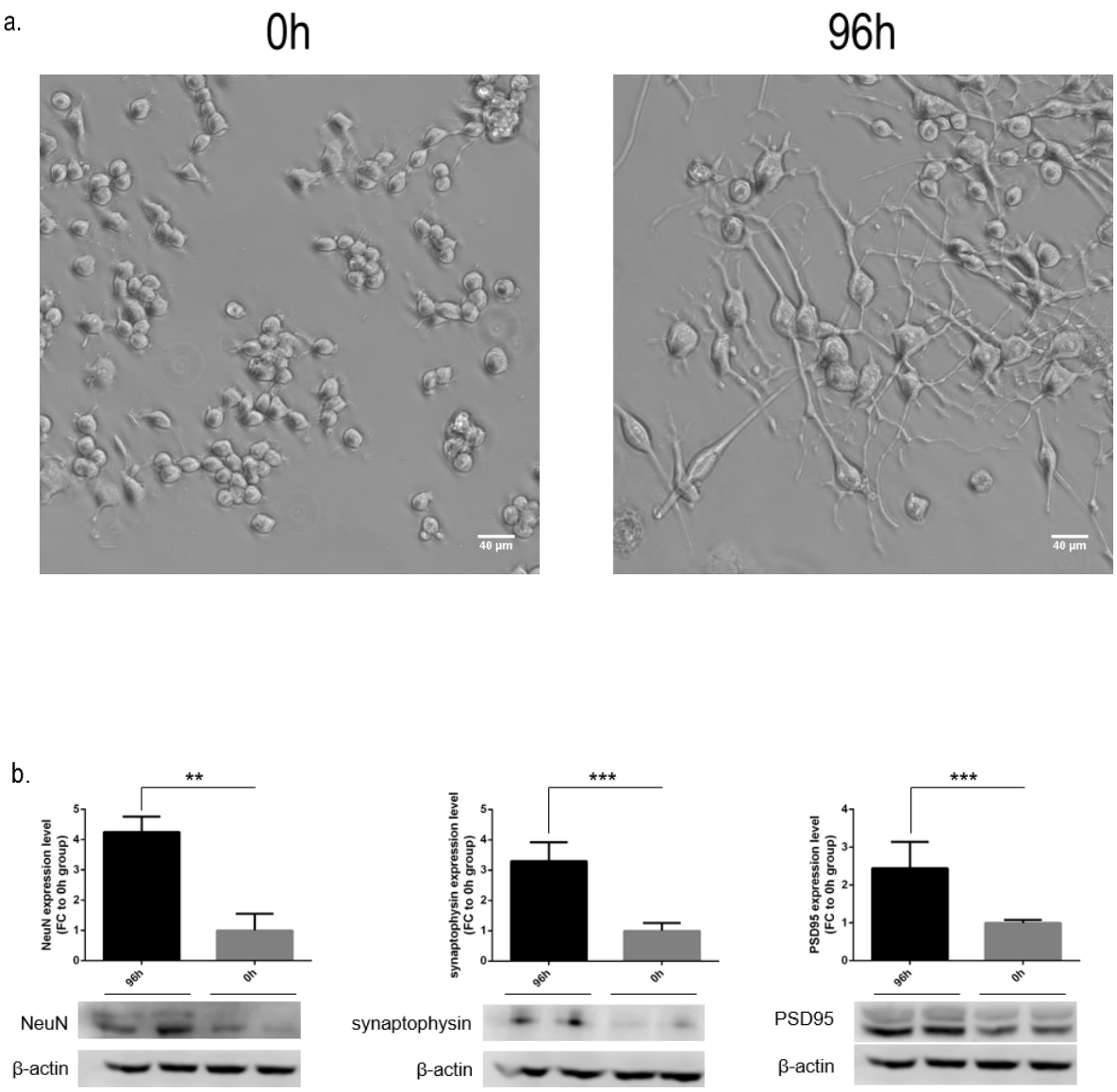
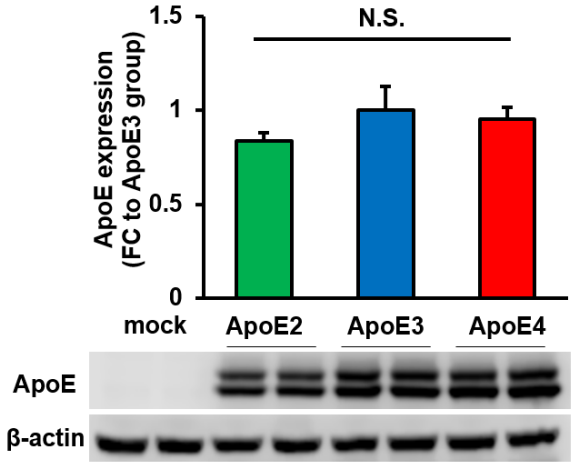


Figure 11. Morphological and immunocytochemical characteristics of 96h RA-induced differentiated Neuro-2a cells. Neuro-2a cells were cultured in medium contain 20 μM retinoic acid for 96h as stated in materials and methods. (a) Cellular morphological alteration by phase contrast images confirmed neuritic processes consistent with neuron-like cells. Scale bars, 40 μm. (b) Several corresponding neuronal biomarkers expression were also significantly elevated compared to undifferentiated cells assessed by western blot. Data were normalized to an internal loading control (β-actin). Immunoblotting results were normalized to undifferentiated group and compared using Student's t-test. **p<0.01, ***p<0.001. Data represent the group mean± SD. n=2-8 per group.

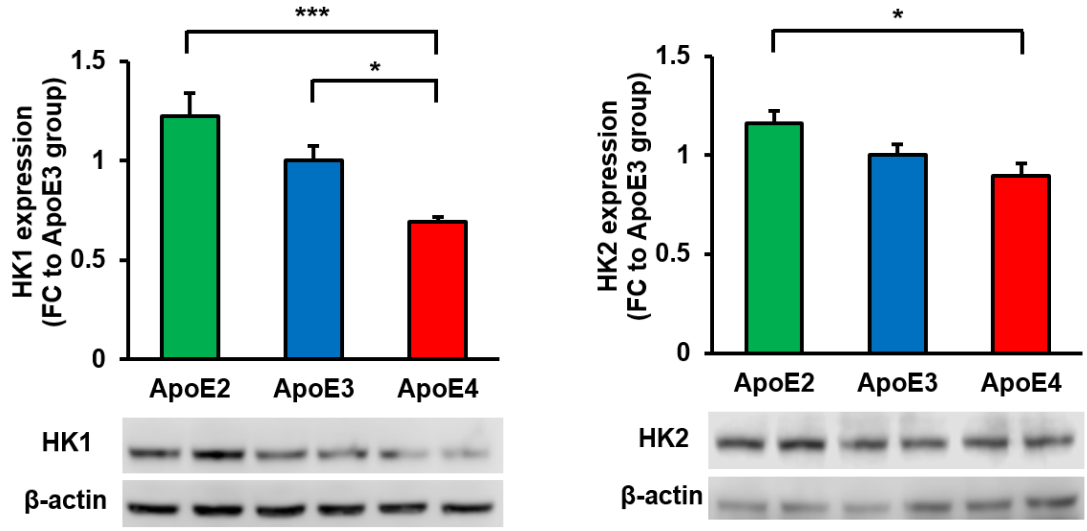
9. Differential regulation of hexokinase and glycolytic function by hApoE2/3/4 also occurred in differentiated N2a cells.

Next, we proceeded to investigate whether the regulation of hexokinase and glycolytic function was also impacted by ApoE isoforms in our in vitro neuronal model. Differentiated N2a cells were transfected with hApoE2/3/4 encoding plasmids or empty vector followed by another 96h incubation. Hexokinase and ApoE protein expression levels were examined using western blot. As shown in Figure 12a, ApoE expression was comparable among the three isoforms, but hApoE2-expressing neurons exhibited a higher level whereas hApoE4-expressing neurons displayed a significantly lower of hexokinase compared to ApoE3 group (Fig.12b). Consistent with the previous data, an elevated level of hexokinase activity and lactate production was observed in hApoE2 transfected neurons. However, hApoE4 transfected neurons demonstrated a deficit compared to the ApoE3 group (Fig.12c). Collectively, these results suggest that in transfected neurons, expression and enzyme activity of hexokinase together with glycolysis are also differentially impacted by ApoE isoforms.

a.


N.S.
ApoE expression (FC to ApoE3 group)
1.5
1
0.5
0
mock
ApoE2
ApoE3
ApoE4
ApoE
β-actin

b.


***
*
HK1 expression (FC to ApoE3 group)
1.5
1
0.5
0
ApoE2
ApoE3
ApoE4
HK1
β-actin
*
HK2 expression (FC to ApoE3 group)
1.5
1
0.5
0
ApoE2
ApoE3
ApoE4
HK2
β-actin

c.

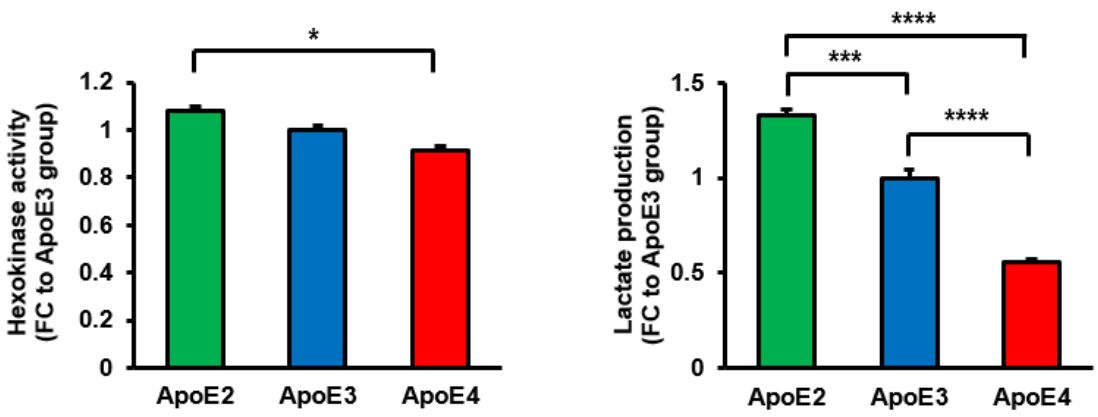

*
Hexokinase activity (FC to ApoE3 group)
1.2
1
0.8
0.6
0.4
0.2
0
ApoE2
ApoE3
ApoE4
****
***
****
Lactate production (FC to ApoE3 group)
1.5
1
0.5
0
ApoE2
ApoE3
ApoE4

Figure 12. Differential regulation of hexokinase and glycolysis by hApoE2/3/4 was also observed in transfected neuronal cells. Differentiated neuronal cells were transfected with human ApoE2/3/4 encoded plasmids or empty vector. (a) ApoE protein expression was confirmed by immunoblotting. The levels were comparable among three transfected groups. (b) Protein expression level of hexokinase was evaluated in transfected neurons using western blot. (c) Hexokinase activity and lactate production were determined by the cell lysate from the transfected neurons and lactate production assay kit. Results were normalized by protein content. hApoE2-expressing cells showed a higher level of both expression and enzyme activity of hexokinase as well as lactate production compared to ApoE4 group. Data were normalized to an internal loading control (β-actin). Results were normalized to ApoE3 group and compared using one-way ANOVA with Tukey's Post hoc Test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Data represent the group mean± SD. n=3-11 per group.

Discussion

Human apolipoprotein E exists mainly in three polymorphic alleles-ε2, ε3 and ε4. Based upon a number of genome wide-association studies, having the ε4 allele is considered the greatest risk factor for late onset Alzheimer's disease, whereas the ε2 allele is believed to be neuroprotective compared to the ε3 allele (Corder et al., 1993; C. C. Liu et al., 2013; Mayeux et al., 1998; Michaelson, 2014; Spinney, 2014; Strittmatter et al., 1993; Ward et al., 2012). Within the past three decades, research efforts have been focusing on the pathological mechanism that underlies the neurotoxicity of ApoE4. However, relatively few studies were carried out regarding the potential neuroprotective role of ApoE2 (L. Wu & Zhao, 2016).

To address this gap, our laboratory initiated a series of studies focusing on the molecular mechanism that underlies the distinctive impacts on AD mediated by the three ApoE isoforms, particularly those that would separate ApoE2 from ApoE3/4. Several studies have demonstrated that before the onset of Alzheimer's clinical symptoms, brain energy hypometabolism plays a crucial role in the preclinical stage, and individuals having ApoE4 exhibit a significant neuronal bioenergetic deficit (Chetelat & Fouquet, 2013; Drzezga et al., 2005; Y. Liu et al., 2016; McGeer et al., 1990; Mosconi, 2005; Mosconi et al., 2006; Smith et al., 1992). Using a qRT-PCR gene array, our laboratory previously demonstrated that hippocampal signaling pathways involved in bioenergetics are differentially regulated by three ApoE isoforms in six-months old hApoE-TR female mice brains. Importantly, IGF-1-mediated signaling activity and downstream glucose

metabolism is significantly elevated in ApoE2 brains compared to ApoE3/4 brains (Keeney, Ibrahimi, & Zhao, 2015).

In another study, the data also indicate that ApoE2 brains exhibit an enhanced synaptic function through the up-regulation of Atp6v subunit expression. Notably, the elevated expression and activity of Atp6v appeared to be directly modulated by glucose metabolism, particularly glycolysis (Woody, Zhou, Ibrahimi, Dong, & Zhao, 2016). In our current study, by using both stable human ApoE cell lines and the neuron model, we confirmed the key step in glycolysis leading to the ultimate functional alteration impacted by the three ApoE isoforms. Moreover, metabolic activity and morphological phenotype associated with overall cell health status in the three ApoE isoforms were further examined. Our data indicate that ApoE isoforms distinctively impact cell health status through the regulation of glycolysis via hexokinase. These findings would provide a potential molecular rationale for the neuroprotective property associated with ApoE2 genotype.

Glucose metabolism is elevated in human ApoE2-expressing neuronal cells via up-regulation of hexokinase

As one of the most metabolically active organs, the human brain requires a large amount of energy to maintain proper function. The human brain consumes approximately 16% of the body's total oxygen, but only accounts for 2% of total body weight (Costantini, Barr, Vogel, & Henderson, 2008). Under normal physiological conditions, glucose is almost the exclusive source of fuel for the central nervous system, whereas the oxidation of fatty acids or ketone bodies are considered minor. The brain's dependence on glucose

puts it at a great risk for neuron loss and declines in cognitive function, if there exists a shortage of glucose supply or deficient ability to utilize glucose. Cerebral hypometabolism during preclinical stage of AD has been extensively suggested, particularly in individuals carrying the E4 allele (Association, 2018; Costantini et al., 2008; Manja Lehmann et al., 2013; M. Lehmann et al., 2014; Y. Liu et al., 2015; Mosconi et al., 2004; Patil, Ballard, Sanchez, Osborn, & Santangelo, 2012; Reiman et al., 1996).

Glucose metabolism happens in three main sequential pathways: glycolysis, citric acid cycle, oxidative phosphorylation, among which glycolysis is an evolutionary ancient pathway providing the subsequent substrates for the afterward steps. One study indicated that aerobic glycolysis is a marker of metabolic functions involved in synaptic plasticity (Vlassenko et al., 2017). It is also reported that decreased aerobic glycolysis associates with a decrease of Aβ in CSF and brain amyloid β accumulation. Moreover, before the onset of cognitive decline in AD, an increased level of key glycolytic enzymes is considered to be beneficial for memory function (R. A. Harris et al., 2016). Another recent study has also revealed evidence in support of the association of glycolytic deficit and high brain glucose with the severity of AD pathology and the expression of AD symptoms (An et al., 2018). Yet the definitive molecular link between abnormal glycolytic function and the severity of AD symptoms or the speed of disease progression is not completely clear. Hexokinase, the first key rate-limiting enzyme in the glycolytic process, phosphorylates glucose to form glucose-6-phosphate in the cytosol. Glucose-6-phosphate is then subject to other catabolic/metabolic pathways, including the remaining steps of glycolysis, the pentose phosphate pathway and conversion to glycogen.

Impaired hexokinase activity in AD patients has been observed in several studies (Harik & LaManna, 1991; Saraiva et al., 2010; Sorbi, Mortilla, Piacentini, Tonini, & Amaducci, 1990). In our study, we confirmed that the regulation of both hexokinase I and II is differentially impacted by the three ApoE isoforms. After validating our stably hApoE-expressing cell lines with a comparable ApoE expression level (Fig.4), we first conducted a time course study to explore the impact by ApoE2/3/4 on hexokinase. Our results suggest that HK expression in the hApoE2/3-expressing stable cell line remained relatively stable, whereas a gradual decrease was observed with increased passages in hApoE4-expressing cells (Fig.5). The data indicate not only a negative but also a slowly progressive impact by ApoE4, highlighting an accumulative property of ApoE4 detrimental effect. Yet whether this feature correlates to the long preclinical stage of AD still remains undefined.

Additionally, we continued other studies among the 3 ApoE isoforms at passage 9, 11 and 13. We found a significantly higher expression and activity of hexokinase in hApoE2-expressing cells compared to hApoE3/4-expressing cells (Fig7a). Functioning as the "pacemaker" of cellular respiration process, dysregulation of hexokinase could lead to multiple energy metabolic abnormalities including both catabolic/anabolic pathways, particularly the glycolytic function. In our study, glycolytic function, reflected by basal glycolysis and glycolytic capacity, is significantly up-regulated in ApoE2 cells, but markedly lower in cells expressing ApoE4 as predicted (Fig.8). Intriguingly, in stable cell lines, we found that as the passage n umber increases, the more remarkable the difference between the lines. We postulate that ApoE4 impairs glycolytic function via the regulation of hexokinase, especially through a chronic pattern that indicates a slow progressive detrimental effect by ApoE4.

It has been reported that pyruvate, NAD^+, NADH, and lactate are held near equilibrium throughout the cell cytosol due to the high activity of lactate dehydrogenase (LDH), and lactate is generated as a main end product of glycolysis (Rogatzki, Ferguson, Goodwin, & Gladden, 2015). Accordingly, lactate production level is often used as an indicator for cellular glycolysis. In our transfected neuronal model, consistent with our observations in stable cell lines, hApoE2-expressing neurons showed a significant higher level of lactate, indicating a robust glycolytic rate, whereas hApoE4-expressing neurons produced a lower level of lactate indicating a relatively weak glycolytic capacity (Fig. 12). However, in addition to the role of hexokinase, several other factors should be considered that might potentially alter the lactate production level as well.

Lactate is produced through the reduction of pyruvate with the concomitant oxidation of NADH to NAD^+, a reaction that requires the catalysis by LDH. A higher expression level and activity of LDH could result in an over production of lactate. The supply of NADH also controls the rate of conversion of pyruvate to lactate. Known as the ox-phos shuttle, the malate–aspartate shuttle and the glycerol–phosphate shuttle together play an important role in maintaining a homeostasis for NADH. When ox-phos shuttle is overwhelmed, high level of NADH will cause an increased production of NAD^+, thus also resulting in an elevated concentration of lactate (Phypers & Pierce, 2006). Moreover, other than hexokinase, the other two key enzymes involved in glycolysis would also determine the production rate of lactate. Phosphofructokinase (PFK) is a key point of glycolysis regulation. It is an important point of allosteric regulation by energy state of the cell and will be inhibited by ATP and citrate. Thus, PFK will be stimulated by the rising of AMP, thus will also cause an increasing of lactate production. The third rate-limiting enzyme,

pyruvate kinase, catalyzes phosphoenolpyruvate to the functional form of pyruvate that the cell requires. It has been found that pyruvate kinase can be regulated by allosteric activation by fructose 1, 6-bisphosphate and inactivation by ATP and alanine (Carbonell, Marco, Felíu, & Sols, 1973). Additionally, the monocarboxylate transporters 4 may also plays a critical role in the efflux of lactate indicated by its high expression in astrocytes. Finally, excessive accumulation of pyruvate could also be induced by the damaged function of the electron transport chain in Kreb's cycle, which in turn would also possibly result in a higher production of lactate.

In the central nervous system, glycolysis is increasingly recognized to play a more and more important role not only in facilitating ATP synthesis driven by synaptic activity together with other cellular process, but also in support of the key function of synaptic and neurite growth and formation (Goyal, Hawrylycz, Miller, Snyder, & Raichle, 2014). When under energy stress, a compensatory mechanism mediated by glycolysis is typically crucial for maintaining enough energy required by endocytosis and synaptic vesicle cycle (Jang et al., 2016). It is reported that glycolysis also plays an important role in preventing synaptic transmission damage under prolonged anoxia (Tian & Baker, 2000).

Recent research found that glycolysis also regulates the assembly, disassembly and function of V-ATPase (Atp6v), a primary pump forming an electrochemical gradient of protons across vesicular membranes, which consists of approximately 20% of total presynaptic protein (Moriyama & Futai, 1990; Woody et al., 2016). Coupled with this concentration process by V-ATPase, neuronal transmitters will be taken up through specific transporters for proper synaptic transmission (Moriyama, Maeda, & Futai, 1992). Increasing evidence links V-ATPase with bioenergetics through the interaction with

glycolytic enzymes. Using an enzyme-linked immunosorbent assay, a-subunit of V-ATPase was confirmed to bind to a rate-limiting enzyme in glycolysis---phosphofructokinase 1 (PFK-1) (Su, Zhou, Al-Lamki, & Karet, 2003). Deletion of Pfk2p was found to alter glucose-dependent V-ATPase reassembly and vacuolar acidification (Chan, Dominguez, & Parra, 2016). Aldolase, an enzyme that catalyzes fructose-1, 6-biphosphate into glyceraldehyde-3-phosphate is found to interact with V-ATPase a, B, and E subunits (Lu, Holliday, Zhang, Dunn, & Gluck, 2001; Lu, Sautin, Holliday, & Gluck, 2004). Nakamura reported that the activation of V-ATPases is involved in a specific signaling pathway that requires PI3K activity, and upon the treatment of 2DG, V-ATPase function can be completely inhibited. (Nakamura, 2004). Notably, a significant V-ATPase disassembly was also observed after inhibition of hexokinase (Kohio & Adamson, 2013). These findings indicate a close relationship between glycolysis and synaptic function, of which a declined level is a hallmark for neurodegenerative disease. Our lab previously demonstrated that ApoE isoforms differentially modulate a specific component of the catalytic domain of Atp6v (Woody et al., 2016). Overall, in this study, we found that glycolytic function is distinctively regulated by ApoE isoforms through hexokinase in both stable cell lines and the transfected neuron model. This up-regulation potentially underlies the neuroprotective property associated with ApoE2 genotype against AD.

In addition to catalytic reactions in the cytosol, we also examined a glucose transporter and perturbed glycolysis in the brain. To date, 14 isoforms of the GLUT/SLC2 have been identified (Thorens & Mueckler, 2009), of which GLUT1-GLUT4 have been widely studied and well-characterized (Bell et al., 1990). Wide spread decline of cortical glucose uptake/metabolism was observed in AD patients, particularly in the parietal and

temporal cortices (De Santi et al., 2001; Friedland et al., 1989; Hoyer, 1991; Jagust et al., 1991; Kalaria & Harik, 1989). Rather than a consequence, this impairment appears to be a cause of neurodegeneration (Hoyer, 2004). GLUT4 functions as the insulin-regulated glucose transporter, mainly responsible for insulin-regulated glucose storage. Despite its primary presence in adipose tissues and striated muscle (skeletal and cardiac), recent research revealed that it is also located in the central nervous system, such as in the hippocampus. Research suggests that it is responsible for the metabolic activities and plasticity of hippocampal neurons, which correlate to depressive-like behavior and cognitive dysfunction (C. C. Huang, Lee, & Hsu, 2010; Patel & Udayabanu, 2014; Piroli et al., 2007). Literature suggests that 27-hydroxycholesterol (27-OH), of which higher level was found in brains and cerebrospinal fluid (CSF) from AD patients, impairs neuronal glucose uptake through an IRAP/GLUT4 system dysregulation (Heverin et al., 2004; Ismail et al., 2017). The term "type-3 diabetes" was even proposed to describe specific abnormalities of insulin signaling in AD brains (Ahmed, Mahmood, & Zahid, 2015; S. M. de la Monte, 2014; Suzanne M. de la Monte & Wands, 2008).

Previous studies indicated a significantly higher level of GLUT4 mRNA in ApoE2 brains compared to Apo3 and ApoE4 brains (Keeney et al., 2015; Long Wu, 2017). On the contrary, in our study we observed no significant difference of GLUT4 protein levels among three ApoE stable cell lines, however, the expression and activity of hexokinase isoforms dramatically varied (Fig.6). A potential explanation would be post-translational modification. Yet several findings indicate that AD-associated pathogenesis is not accompanied by the reduction of GLUT4 expression or IDE, suggesting that other signaling pathways are involved in insulin/IGF-I mechanisms (Ying Liu, Liu, Iqbal,

Grundke-Iqbal, & Gong, 2008; Steen et al., 2005). GLUT1 and GLUT3, however, were observed to be significantly reduced in the brains of AD patients (I. A. Simpson, Chundu, Davies-Hill, Honer, & Davies, 1994; Ian A. Simpson et al., 2008; Szablewski, 2017; Winkler et al., 2015). Down-regulation of GLUT1 exacerbates Alzheimer's disease vasculoneuronal dysfunction (Winkler et al., 2015) consistent with its crucial role on BBB. Diminished GLUT3 expression, mostly presenting in neuronal axons and dendrites, could render the brain more vulnerable to AD (An et al., 2018). Deficiency of both GLUT1 and GLUT3 was reported to impair cerebral glucose metabolism and contribute to neurodegeneration, particularly through down-regulation of O-GlcNAcylation and hyperphosphorylation of tau in AD (Ying Liu et al., 2008). While the mechanism by which signaling pathways regulate brain glucose homeostasis as well as severity of AD pathology and clinical symptom remain undefined, improving glycolytic function via hexokinase and/or GLUT expression/function would set up an underlying neuroprotective mechanism against AD.

Up-regulation of glycolysis underlies a neuroprotective property of ApoE2

An increasing body of evidence shows that brain develops pathological lesions many years before clinical symptoms manifest (Braak & Braak, 1991; Dubois et al., 2016; Morris et al., 1996; R. Sperling, Mormino, & Johnson, 2014). By using positron emission tomography with 2-[18F] fluoro-2-deoxy-D-glucose (FDG–PET), a severe reduction of the cerebral metabolic rate for glucose (CMRglc), which may contribute to the gradual cognitive decline, was observed during this long period of preclinical stage (Mosconi, 2013). A comparatively steep loss of cognition, which is the hallmark of AD, will take

place after reaching a reflection point under the long pathophysiological change, including significant impairment in synaptic plasticity and transmission, lower synaptic proteome as well as declined synaptic density (Counts, Nadeem, Lad, Wuu, & Mufson, 2006; Klein, Mace, Moore, & Sullivan, 2010; Scheff, Price, Schmitt, DeKosky, & Mufson, 2007; P. T. Xu et al., 2006). The ApoE4 genotype has long been implicated to be associated with perturbed energy metabolism and accelerated cognition decline in preclinical stage of AD (Bonham et al., 2016; Klein et al., 2010; Thai et al., 2015). In line with these findings, results of our study indicate a differential overall metabolic activity impacted by three ApoE isoforms, with ApoE2 cells exhibiting the highest while hApoE4 displayed the lowest profile (Fig.9a). One study previously discovered 50× more neurons and 25× more glial cells with nuclear DNA fragmentation in the brains of patients with AD than in non-demented controls (Bancher, Lassmann, Breitschopf, & Jellinger, 1997). In support of this notion, morphological difference was also observed among three stable cell lines, where ApoE2 cells were more likely to exhibit heathier regular shape while ApoE4 cells had a relatively higher tendency to get swollen and aggregate into clumps (Fig.9b). These findings suggest a potential mechanism that renders ApoE4 cells more vulnerable compared to the other two isoforms.

Neurodegenerative disease, such as AD, is characterized by gradual loss of function together with the integrity of neuronal cells in regions involved in memory and learning ability. Progression of the disease will ultimately lead to neuron death. As more and more neurons are lost, patients become progressively worse and die within 5-20 years after the first diagnosis. Evidence showed DNA damage, nuclear apoptotic bodies, chromatin condensation, and the induction of selected apoptotic genes. Multiple cell death pathways,

especially correlated to either the Aβ hypothesis or tau hyperphosphorylation, have been explored in the past decades, of which caspase (Casp)-dependent and autophagy pathways have been widely implicated in AD pathophysiology (Cotman Carl & Su Joseph, 1996; Cotman, Poon, Rissman, & Blurton-Jones, 2005; Dickson, 2004; Guo et al., 2004; Martinez-Vicente & Cuervo, 2007; Piras, Collin, Gruninger, Graff, & Ronnback, 2016; Rohn & Head, 2008). Here, in our study, overall cell apoptosis was not observed among three ApoE isoforms within the passages studied (Fig.10). We postulate that unlike previous work our cell model is ApoE isoform dependent without intervention of either Aβ or tau. Second, neuron death may only happen in the late stage of AD and the initiation of a cell death cascade requires an accumulation of energy deficits mediated by ApoE4 to reach the reflection point, highlighting the ApoE role before the onset of cell death. While in-depth investigation of the underlying mechanism is warranted, up-regulation of glycolysis through hexokinase by ApoE2 could potentially help improve brain energy metabolism, thus delay the progression of synaptic loss or initiation of neuron death.

There are some limitations of our studies, which could be addressed in the future. Lactate is a product of a series catalytic reactions associated with multiple factors. Even though previous gene profiling data didn't indicate a significant difference among the enzymes other than hexokinase, which was differently impacted by ApoE isofroms in ApoE-TR mice brains, the downstream protein expressions and functions were still worth an exploration. In the first cell model, all corresponding experiments were based on one valid set of stable cell lines. Controls and more replicated experiments based on different cell lines need to be conducted. As for the imaging analysis, in this study, the imaging data were initially provided to further illustrate the morphological phenotype of the cells, thus

the further qualification of the images and statistical analysis were not done accordingly and will be done in the future in order to correctly correlate the imaging assays with the morphological phenotype of the cells. Moreover, all current studies were limited to *in vitro* studies. To circumvent the limitation of the experimental model, both *in vitro* and *in vivo* animal models as well as experimental paradigms need to be developed and characterized in the future.

In summary, our research examined glycolytic differences mediated by three ApoE isoforms, with our primary focus on the potential neuroprotective property of ApoE2 as well as the neurotoxicity by ApoE4. The data indicated that three ApoE isoforms differentially regulate glycolysis through hexokinase, with ApoE2 cells exhibiting a most robust profile, whereas ApoE4 cells are the most deficient, both in stable cell lines and the transfected neuron model. Notably, consistent with previous findings, this regulation by ApoE isoforms is independent of GLUT4, a glucose transporter that responds to insulin signaling. Moreover, the functional difference mediated by ApoE isoforms was reflected by glycolysis and glycolytic capacity. Since data suggested robust glycolysis parameters in hApoE2-expressing cells, while hApoE4-expressing cells are deficits, we further hypothesized that the differential glycolytic function mediated by three ApoE isoforms is directly related to metabolic activity and cell health status. Cell health status was evaluated through overall metabolic activity and morphological phenotype. In line with previous findings, ApoE2 cells exhibited a higher level of metabolic activity and appeared healthier, while ApoE4 cells demonstrated a lower level and less regular morphology. Particularly, no significant difference was found in BAX expression among three ApoE stable cell lines within studied passages indicating a period before the initiation of cell death cascade.

Targeting at this period with strategies for introducing an ApoE2-mediated neuroprotective mechanism to improve neuron energy metabolism and viability hold promise for elevating brain resilience against AD and delaying or even halting the progression of AD.

Conclusions

Findings of this study provide insight to how human ApoE isoforms may affect overall metabolic activity as well as cell health status through the regulation of glycolysis via hexokinase. Our results illustrate that ApoE4-mediated detrimental effects on glycolysis was displayed in a chronic pattern indicating its cumulative impact on the cells. By contrast, ApoE2-expressing cells remained robust within the passages studied, both in expression and activity of hexokinase as well as glycolytic function compared to ApoE4 groups. According to our study, this ApoE2-mediated robustness directly correlates to healthier cell status and higher metabolic activity independent of apoptotic process and insulin-regulated glucose transport step. These results further highlight the important role of glycolysis in maintaining resilience of cells, which could set a foundation for neuroprotective/neurodegenerative mechanism mediated by ApoE isoforms in the development of AD. Moreover, in line with our findings in ApoE-expressing stable cell lines, we also observed differential regulation of hexokinase and glycolysis by three ApoE isoforms in our differentiated neuronal model. This helps further confirm that glycolytic regulation by ApoE plays an important overall role in the central nervous system. Taken together, while more advanced studies on the potential signaling pathways involved and in vivo experiments need to be conducted, our study showed that human ApoE isoforms distinctively impact brain cells overall metabolic activity and health status in a chronic pattern in which ApoE2-mediated the neuroprotective effect may be largely induced through the up-regulation of glycolytic function.

Future directions

Based on current findings, a considerable amount of studies are warranted. Glucose transporters exist in several isoforms, which renders the single study of GLUT4 a certain extent of limitation. GLUT1-GLUT4 have been well-characterized by extensive studies, of which glucose transporter type 3 is abundantly expressed in neurons and has a higher affinity for glucose (Bell et al., 1990; Navale & Paranjape, 2016). It is also reported that GLUT1 and GLUT3 are markedly decreased in Alzheimer's patients, especially in the cerebral cortex (Szablewski, 2017). More studies could be conducted to further explore the potential role of ApoE on GLUT in AD.

ApoE and ApoE derived-peptides have been associated with multiple signaling pathways (Fig.13) (Zhou, 2013). Specifically, hexokinase has been reported to be involved in Akt, Wnt signaling pathways and function as a downstream regulator of glycogen synthase kinase-3 (GSK-3), which is potentially relevant to neurodegenerative disease (Arboleda et al., 2007; Cisternas, Salazar, Silva-Alvarez, Barros, & Inestrosa, 2016; Duarte, Santos, Oliveira, Santos, & Rego, 2008; Gimenez-Cassina, Lim, Cerrato, Palomo, & Diaz-Nido, 2009; Regenold et al., 2012). Future experiments should be carried out to gain an in-depth understanding on the molecular mechanism by which ApoE isoforms modulate glycolytic function via the regulation of hexokinase.

Additionally, further in vivo investigations are necessary to fully illustrate the ApoE impact on cognitive function and memory loss. In our previous study, we confirmed that glycolysis may impact many other downstream pathways independent of energy production yet are critical for neuronal function, such as glycolytic regulation of synaptic

V-ATPase that would ultimately lead to an impact on synaptic transmission. The mechanism underlies how these glycolytic deficits link to the severity of AD or to its rapid onset will be further investigated in my PhD study.

Our ultimate goal for all these studies is to find a therapeutic intervention that could prevent or modify the disease by promoting the resilience and plasticity of the brain against AD. Results shown here suggest that ApoE2-mediated up-regulation of glycolysis may promote overall metabolic activity and cell health status compared to ApoE4. Accordingly, we hypothesize that introduction of ApoE2 gene/protein into ApoE4-expressing or AD brain would enhance glycolytic metabolism and neuronal viability against neurodegenerative stress. Future studies will focus on evaluating the bioenergetic, synaptic, and viability changes in ApoE4-expressing neuronal cells induced by introduction of ApoE2 gene under both normal and oxidative stress conditions. Bioenergetic and cognitive changes caused by the delivery of human ApoE2 protein in the brains of human ApoE4 gene-targeting replacement mice and transgenic AD mice under the facilitation of E-cadherin peptides will also be investigated.

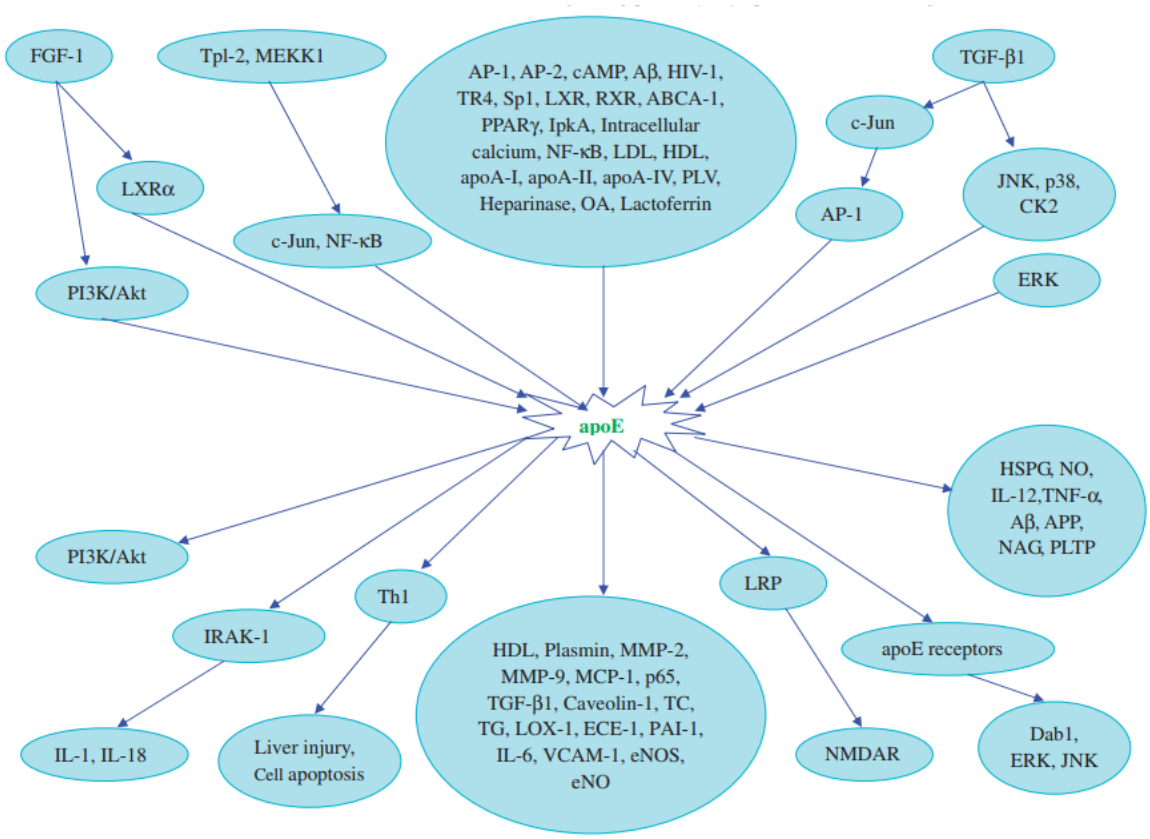


Figure 13. Signaling pathways for ApoE. Adapted from Zhou, T. B. (2013). Signaling pathways of ApoE and its role of gene expression in glomerulus diseases. J Recept Signal Transduct Res, 33(2), 73-78. doi:10.3109/10799893.2013.765466.

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